AGING AND N-METHYL-D-ASPARTATE RECEPTORS

By

JOSEPHINE JEAN MITCHELL

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1995

To Dad

ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. Kevin J. Anderson, for sharing his extensive knowledge and for his support throughout my doctoral studies. My research was supported by a National Institute of Aging grant (NIA #AG08843). I would also like to recognize my other committee members: Dr. A. John MacLennan, Dr. Joanna Peris, Dr. Tom Vickroy and Dr. Don Walker for their constructive criticisms and suggestions. I am also very grateful to Dr. Janet Zengel's for her support and friendship through the years.

Michael S. Sapper deserves a special acknowledgement for his technical assistance as well as his friendship from the very beginning. Special thanks go out to Tanya McGraw for her friendship and support as well as to many of my other fellow graduate students.

I wish to especially mention my father, Ignaceous J. Maddalena, who unfortunately passed away just before I began my doctoral studies. He was and always will be the person who fueled my love for academics and taught me the importance of hard work and perseverance in the pursuance of knowledge. My mother, Helen, has been a loving and supportive figure throughout my life and I wish to thank her with all of my heart as well as the rest of my family and friends.

Finally, and most importantly, I wish to acknowledge my husband, Thomas Mitchell (Mitch), who has given me invaluable support throughout my academic pursuits as the 'one and only love of my life' and father to our two beautiful sons Kyle Ryan and Ty Joseph. I could never have achieved all I have worked for without the constant love and support of my family and friends.

TABLE OF CONTENTS

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

AGING AND THE N-METHYL-D-ASPARTATE RECEPTOR

By

Josephine Jean Mitchell

December, 1995

Chairman: Kevin J. Anderson
Major Department: Neuroscience

Aging is associated with a reduction in cognition and memory in humans and other animals. Excitatory amino acids (EAA) play pivotal roles in learning and memory. L-Glutamate is thought to be the major EAA transmitter in mammalian brain. L-Glutamate transmitter systems may therefore be involved in age-related deficits. The N-methyl-D-aspartate (NMDA) ionotropic glutamate receptor subtype appears important in learning and memory. It is critical to long-term potentiation and NMDA antagonists impair performance of rats in spatial and reference memory tasks. Antagonism of NMDA receptor-mediated neurotransmission produces behavioral deficits strikingly similar to those detected in aged animals.

Thus deficits in NMDA receptor neurotransmission may underlie age-related changes in neuronal plasticity. One possible way NMDA neurotransmission could be reduced in aged animals is by alterations in NMDA receptor/channel complexes.

The central hypothesis tested in this study was that there are measurable, anatomically specific age-related changes in the NMDA receptor and its individual subunits. An initial examination of age-related differences in NMDA receptor density in brains of 6-, 12- and 24-month-old F344 rats was performed with [^{3}H]MK-801 *in vitro* autoradiography. An age-related decrease was found in the entorhinal and inner frontal cortices and the lateral striatum.

An analysis of L-glutamate's ability to enhance [^{3}H]MK-801 binding to NMDA receptor channels was performed by varying L-glutamate concentration. E_{max} and EC_{50} values were obtained for several brain regions showing an age-dependent decrease in E_{max} values without changes in EC_{50}.

The possibility that receptor binding changes were due to decreases in specific NMDA receptor subunits was analyzed using *in situ* hybridization. A significant difference was seen in NMDAR1 subunit mRNA in all brain regions. Splice variants $NR1_{0xx}$, $NR1_{1xx}$ and $NR1_{x1x}$ changed in fewer regions while NMDAR2 subunits did not change with age.

No age-related differences in NMDAR1 and NMDAR2A/B protein density levels were found using immunocytochemistry.

These results indicate the NMDA receptor undergoes regionally specific changes during aging and these changes

may account for some of the cognitive deficits in the aging population.

CHAPTER 1
INTRODUCTION

Background

Ramon y Cajal, as early as the turn of the century, proposed the connective foundation of neural memory (Cajal, 1911). Many years later, Hebb (1949) formulated his principles of memory formation which was based upon facilitation of contacts between neurons. Both Cajal and Hebb focused on the synapse as the location of plastic change in the formation of memory. They also theorized that the processes of learning and memory result from changes in the strength of synaptic transmission (Cajal, 1911; Hebb, 1949).

Bliss and Lomo (1973) found that in the hippocampus, a brain region known to be important for learning (Nicoll et al., 1988), brief repetitive activation of excitatory pathways resulted in a substantial increase in synaptic strength that lasted for many hours and, *in vivo*, even for weeks. Since its initial discovery, this synaptic enhancement, referred to as long-term potentiation (LTP), has provided a model in vertebrate brain for a cellular mechanism of learning and memory (Bliss and Lomo, 1973). LTP is defined as an electrophysiological phenomenon of persistent change in synaptic strength or efficacy as a result of

impulse transmission across synapses. These activity-induced changes in the efficacy of existing synapses are thought to be mediated by excitatory synaptic transmission throughout the central nervous system (Bliss and Lomo, 1973).

Excitatory glutamatergic synapses are abundant throughout the central nervous system (CNS), especially in the hippocampus and cerebral cortex (Westbrook and Jahr, 1989). There are at least three types of ionotropic glutamate receptors classified by the potent, selective agonists N-methyl-D-aspartate (NMDA), kainate (KA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) (Foster and Fagg, 1984). During low-frequency synaptic transmission, glutamate is released from the presynaptic terminal and acts on both the NMDA receptor subtypes as well as the non-NMDA receptor subtypes (KA and AMPA). AMPA and KA receptors are permeable to K^+ and Na^+ and provide a voltage-independent means of depolarizing the postsynaptic neuron. However, the NMDA receptor has unique biophysical characteristics making it highly relevant to neural plasticity and memory formation (Collingridge, 1987; Cotman and Iversen, 1987; Harris et al., 1984). During low frequency stimulation, ion flux through the NMDA channel is blocked by Mg^{+2} in a voltage-dependent manner. Mg^{+2} ions are expelled from membrane channels after the postsynaptic membrane has reached a certain level of depolarization. Following relief of the Mg^{+2} block, the receptor channel is permeable to Na^+ and Ca^{+2}. Furthermore, NMDA receptor-

mediated current increases as a function of the degree of depolarization are longer in duration than those from non-NMDA receptors. The necessity for both presynaptic release of transmitter to bind to the NMDA receptor as well as sufficient postsynaptic depolarization to relieve the voltage-dependent channel block by Mg^{+2} give this receptor Hebb-like properties.

It is now thought that the activation of NMDA receptors is responsible for the induction of LTP in the hippocampus (see Nicoll et al., 1988 for review). NMDA antagonists have been shown to block the development of LTP in the CA1 region of the hippocampus during high-frequency tetanic stimulation of the Schaffer commissural pathway (Collingridge et al., 1983) as well as LTP in the dentate gyrus after perforant path stimulation (Errington et al., 1987). In addition, a variety of NMDA antagonists, both competitive (e.g., 2-amino-5-phosphonovalerate [AP5], CGS 19755) and noncompetitive (e.g., MK-801, phencyclidine [PCP]), have been shown to impair performance of rats in the acquisition of spatial working and reference memory tasks (Shapiro and Caramanos, 1990). NMDA receptor channel antagonism by MK-801 also impairs performance of rats in aversively motivated complex maze tasks (Spangler et al., 1991). These results provide evidence for the NMDA receptor playing a role in memory and spatial learning and LTP (see also Danysz et al., 1988; Morris et al., 1986).

Aging is associated with a reduction in cognition and memory in humans as well as other animals (Barnes, 1979; Barnes and McNaughton, 1985). Senescent rats are slower than young rats in learning various spatial tasks (Barnes, 1979; DeToledo-Morrell et al., 1984) which may be due to age-related changes in NMDA receptor-mediated neurotransmission. In support of this, it has been shown that antagonism of NMDA receptor-mediated neurotransmission produces behavioral deficits strikingly similar to those detected in aged animals (Bonhaus et al., 1990).

Taken together, the above evidence suggests that a reduction in NMDA receptor-mediated neurotransmission may underlie age-related changes in neuronal plasticity. One means by which NMDA neurotransmission could be reduced in aged animals is by an alteration in the NMDA receptor/channel complex itself.

Previous Studies Examining Age-Related Changes in NMDA Receptors

Previous studies have examined the effects of aging on the density of central excitatory amino acid (EAA) receptors in brain membrane preparations. For example, Tamaru et al. (1991) reported a reduction in the number of NMDA receptors in the cerebral cortex and hippocampus of aged rats. Specifically, they found a significant decrease in [^{3}H]glutamate binding displaceable by NMDA, in strychnine-insensitive [^{3}H]glycine binding and in [^{3}H]MK-801 binding in

both brain regions. Scatchard analysis revealed that the reduction was due to a decrease in the number of binding sites, not to an alteration in affinity. The reduction in the number of receptors was apparently not due to a relative increase of insoluble protein in membrane fractions since the protein concentrations and ratio of protein concentration to tissue wet weight were not significantly different between young and aged animals. Similar results were obtained by Ingram et al. (1992), who observed a marked (>50%) age-related reduction in NMDA receptor binding in rat hippocampal brain homogenates, and by Peterson and Cotman (1989) who found reductions in NMDA-displaceable [^{3}H]L-glutamate binding density in two strains of aged mice.

The effect of increasing age on the binding of [^{3}H]L-glutamate to NMDA receptors in the brain of the BALB/c and C57B1 mouse strains was also determined using *in vitro* quantitative autoradiographic analysis (Magnusson and Cotman, 1993). In this study, a significant decrease in binding to NMDA receptors occurred with increasing age (ranging from 3 through 30 months). NMDA receptors, as opposed to non-NMDA receptors, were selectively affected by age-related changes in the majority (17 of 20) of the cortical, subcortical and hippocampal regions assayed in both strains of mice. [^{3}H]Kainate and [^{3}H]AMPA (non-NMDA) binding, on the other hand, was decreased in only 7 of 21 and 4 of 21 regions, respectively. They concluded that the NMDA receptor is

selectively vulnerable to the aging process throughout most cerebral cortical, subcortical and hippocampal regions.

A similar autoradiographic study has also demonstrated that specific brain regions in aged animals exhibit a decline in NMDA receptor density (Anderson et al., 1989). The density of NMDA receptors labeled with [^{3}H]L-glutamate in young-adult (4-month-old) Fischer 344 rats was compared to aged (24- to 26-month-old) rats. The areas that showed a greater than 30% decrease in aged rats included the lateral striatum, inner layers of the entorhinal cortex and the lateral septal nucleus. The receptor density in inner parietal cortex and anterior cingulate cortex was decreased by 10% in aged rats when compared to young-adults. Within a given brain region there was no significant difference in the affinity (K_D) of the NMDA receptor for [^{3}H]L-glutamate. However, a significant decrease in the B_{max} was seen within the brain regions most affected by increasing age which indicated that there was a decrease in the total number of receptors (Anderson et al., 1989). This group also utilized [^{3}H]glycine binding to probe the associated allosteric activating site on the NMDA receptor. Areas that showed the greatest degree of loss of [^{3}H]glycine binding included the lateral striatum, parietal cortex and the entorhinal cortex. There was no significant decrease in the lateral septal nucleus and the loss of glycine binding sites in aged rats was more profound in the parietal cortex and outer portions

of the entorhinal cortex when compared to [^{3}H]L-glutamate binding.

Age-related changes in the glutamatergic neurotransmitter systems in rats and rhesus monkeys has been examined utilizing NMDA-displaceable [^{3}H]L-glutamate binding to brain homogenates (Wenk et al., 1991). [^{3}H]L-Glutamate binding density was decreased in many brain regions in aged rats (24 month-old) when compared to young (5 month-old) rats. Specifically, the sensory-motor cortex, the parietal-occipital cortex, the hippocampus and the caudate nucleus all showed a significant decrease in binding density. In monkey brains, NMDA-displaceable [^{3}H]L-glutamate binding was decreased in most brain regions analyzed and particularly noticeable in the frontal and temporal lobes of the aged monkeys (29-34 years) when compared to young (4-9 years) monkeys.

Senescence-accelerated mice (SAM-P/8) have been used as a murine model of aging and memory dysfunction (Kitamura et al., 1992). This strain of mice shows an age-related deterioration of learning and memory at an earlier age when compared with control mice. In brain homogenates of hippocampus and cerebral cortex from SAM-P/8 mice there was a significant increase in the content of glutamate and glutamine when compared to controls. Potassium-evoked endogenous glutamate release from the brain slices of SAM-P/8 mice was increased in comparison with the control strains at 9 and 11 months. Additionally, the B_{max} of [^{3}H]dizocilpine

(MK-801) binding in the cerebral cortex was decreased in SAM-P/8 but not in controls. This suggests that synaptic dysfunctions in the glutamatergic system occur in the CNS of the SAM-P/8 mouse strain (Kitamura et al., 1992).

Molecular Cloning and Characterization of the NMDA Receptor/Channel Complex

A functional cDNA clone for the rat NMDA receptor (NMDAR1) was first isolated in 1991 (Moriyoshi et al., 1991). The single protein encoded by the cDNA was shown to form a functional receptor/ion-channel complex with electrophysiological and pharmacological properties characteristic of the NMDA receptor; i.e. agonist and antagonist selectivity, modulation by glycine, Ca^{2+} permeability, a voltage-dependent channel block by Mg^{2+}, and Zn^{2+} inhibition (Moriyoshi et al., 1991).

Three cDNAs encoding different NMDA receptor subunits were isolated by polymerase chain reaction (PCR) (Monyer et al., 1992). Two degenerate oligonucleotide primers were designed after largely conserved peptide sequences in ionotropic EAA receptor subunits, with which NMDAR1 shares several small sequence islands around putative transmembrane (TM) segments. These primers were used to PCR amplify homologous sequences from rat brain cDNA. Three full-length cDNAs, having sequences identified from the PCR products, were named NMDAR2A (NR2A), NR2B and NR2C. The predicted proteins were between 55% (NR2A and NR2C) and 70% (NR2A and

NR2B) identical to each other, but were only about 20% identical to homologous AMPA-selective glutamate receptor subunits (GluRs) and NMDAR1 (Monyer et al., 1992).

The functional properties of the expressed NR2 subunit were examined using a *Xenopus* oocyte expression system. No detectable calcium currents were recorded after bath application of glutamate or NMDA to oocytes expressing one or two NR2 subunits, which indicated that NR2 subunits may not form functional homomeric or heteromeric channels. However, large currents were measured in oocytes coexpressing NR1 and any one of the NR2 subunits. On average, the NMDA-induced currents in oocytes expressing NR1 and NR2A, NR2B or NR2C were 100 times larger than they were in oocytes expressing homomeric NR1 channels. These currents also more closely resemble native NMDA receptors. This indicated that heteromeric configurations are likely to form from NR1 subunits and members of the NR2 subunit family (Monyer et al., 1992).

NMDAR1 and NMDAR2 subunits carry an asparagine residue in the putative channel forming region TMII, whereas a glutamine or arginine residue resides in the homologous position of the AMPA-selective glutamate receptor subunits (Nakanishi et al., 1992). The importance of the asparagine residue in the regulation of Ca^{+2} permeability and channel blockade was shown by electrophysiological characterization of receptors in which the asparagine was replaced with either glutamine or arginine (Nakanishi et al., 1992). These

substitutions reduced or abolished Ca^{+2} permeability and inhibition by Mg^{+2}, Zn^{+2}, and MK-801. Thus, this particular asparagine residue may constitute a distinctive functional determinant in subunits belonging to the NMDA receptor (Monyer et al., 1992).

Previous *in situ* hybridization studies have revealed that NMDAR1 messenger RNA (mRNA) in adult rat brain is expressed in almost all neuronal cells throughout the brain (Moriyoshi et al., 1991). This group observed prominent expression of NMDAR1 mRNA in the cerebellum, hippocampus, cerebral cortex and olfactory bulb. High expression was also seen in the granular layer of the cerebellum, in granule cells of the hippocampal dentate gyrus and in pyramidal cells throughout hippocampal areas CA1-CA4.

The anatomical distribution of mRNA for NMDAR2 subunits has been examined with *in situ* hybridization (Buller et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monaghan et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991; Nakanishi, 1992). NMDAR2A mRNA is widely expressed in many brain regions, and this expression is prominent in the cerebral cortex, hippocampus, olfactory bulb, some thalamic nuclei, pontine nuclei, inferior olivary nuclei and cerebellar cortex. The NMDAR2B mRNA expression is prominent in most of the telencephalic and thalamic regions but relatively low in the hypothalamus, cerebellum and lower brain stem. The distribution of the NMDAR2C mRNA is more discrete; its expression is extremely high only in the

granular layer of the cerebellum. NMDAR2D mRNA is mainly expressed in the diencephalic and lower brain stem regions.

Recently, the distribution of NMDA receptor subtypes, as identified with autoradiography, have been shown to correspond to specific receptor subunits (Monaghan et al., 1993). Early studies had indicated that there were two populations of NMDA receptors; agonist-preferring (striatal-type) and antagonist-preferring (thalamic-type) (Monaghan et al., 1988). These populations were classified by whether they displayed a higher affinity for agonist or for antagonist. Monaghan (1991) showed that low concentrations of L-glutamate selectively promoted the binding of [^{3}H]MK-801 to striatal-type NMDA sites. He concluded that low concentrations of L-glutamate appeared to preferentially activate striatal NMDA receptors without activating thalamic and cortical NMDA receptors. Conversely, with higher concentrations of L-glutamate, [^{3}H]MK-801 will also label NMDA receptors of the lateral thalamic nuclei and cerebral cortex (Monaghan and Anderson, 1991).

More recently, two other pharmacologically-distinct populations of NMDA receptors have been defined by autoradiography. These receptors were identified in the midline thalamic nuclei and in the cerebellum (Monaghan et al., 1993). It has been shown that the anatomical distribution of each of these four native receptor populations corresponds to a specific NMDAR2 subunit (Monaghan et al., 1993). The NMDAR2A transcript has a

distribution very similar to the "antagonist-preferring" NMDA receptor subtype. The NMDAR2B subunit mRNA is the only NMDAR2 species found in regions enriched in "agonist-preferring" sites. NMDAR2C subunits are largely restricted to the cerebellum which contains a pharmacologically-distinct receptor subtype and the NMDAR2D subunits are restricted to the midline-thalamic nuclei NMDA receptor subtype (Monaghan et al., 1993). These data suggest that NMDAR2 subunits may contribute to the pharmacological diversity of native NMDA receptors.

Alternative splicing has been shown to generate several functionally distinct NMDAR1 subunits (Anatharam et al., 1992; Durand et al., 1992; Nakanishi et al., 1992; Sugihara et al., 1992). Structures and properties of seven isoforms of the NMDAR1 receptor are differentiated from each other by an insertion at the extracellular amino-terminal regions or deletions at two different carboxy-terminal regions, or by combinations of the insertion and deletions. All of these isoforms have been shown in the *Xenopus* oocyte expression system to induce electrophysiological responses to NMDA and respond to various antagonists selective to the NMDA receptor (Sugihara et al., 1992). The nomenclature of Durand et al. (1993) denotes each NMDAR1 splice variant by the presence or absence of the three alternatively spliced exons in the 5' to 3' direction. A subscripted $_0$ denotes exclusion of an exon while a subscripted $_1$ denotes its inclusion. For example,

$NR1_{111}$ has all three exons, $NR1_{000}$ has none, and $NR1_{100}$ has only the N-terminal insert (Figure 1-1).

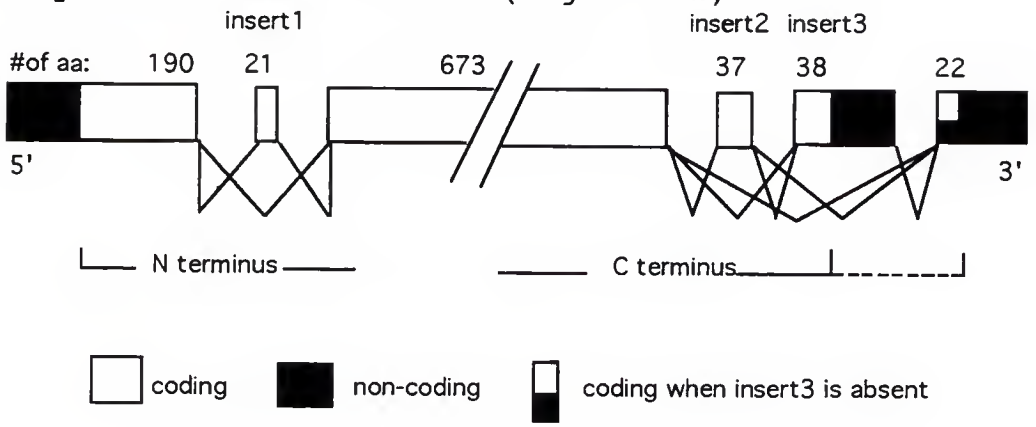


Figure 1-1. Proposed gene structure of the NMDAR1 receptor (Durand et al., 1993). Three putative inserts can be spliced in or out to form the mature mRNA. The NMDAR1 splice variants are denoted by subscripts indicating the presence or absence (1 or 0) of the three inserts in the 5' to 3' direction.

A recent study by Buller et al. (1994) described the anatomical distributions of $NMDAR1_{1xx}$ (the NMDAR1 splice variant containing insert 1) and $NMDAR1_{0xx}$ (the NMDAR1 splice variant lacking insert 1). They found $NMDAR1_{1xx}$ mRNA density varied across cortical regions with the parietal, temporal, and superficial entorhinal cortices displaying threefold higher levels of $NMDAR1_{1xx}$ mRNA than the anterior cingulate, perirhinal, and insular cortices. In contrast, higher levels of $NMDAR1_{0xx}$ mRNA were found in the anterior cingulate, perirhinal, and insular cortices. They concluded that the localization of $NMDAR1_{1xx}$ and $NMDAR1_{0xx}$ mRNA between cortical regions paralleled the distribution of antagonist-preferring and agonist-preferring NMDA receptors, respectively.

$NMDAR1_{1xx}$ mRNA displayed a lateral-to-medial gradient pattern within the striatum whereas $NMDAR1_{0xx}$ was shown to be moderately higher (15%) in the medial striatum than the lateral striatum. $NMDAR1_{1xx}$ was present in low levels throughout the septum while high levels of $NMDAR1_{0xx}$ mRNA were found in this region. $NMDAR1_{1xx}$ mRNA was present at high levels throughout the thalamus with low levels of $NMDAR1_{0xx}$. Overall, this study showed that agonist-preferring NMDA receptors are found predominately in the subset of brain regions that contain both NMDAR2B and $NMDAR1_{0xx}$ mRNA whereas the antagonist-preferring NMDA receptors are found predominately in brain regions containing both NMDAR2A and $NMDAR1_{1xx}$ mRNA. Monaghan and Buller (1994) also found that NMDAR1 mRNAs that are alternatively spliced at the second and third inserts have distribution patterns that are dissimilar to that of previously described NMDA receptor subtypes. No differences in NMDA receptor pharmacological properties have been found after studying homomeric receptors of NMDAR1 mRNA that contain alternatively spliced exons at the second and third insert sites (Durand et al., 1993; Nakanishi et al., 1992). Because of these findings, alternative splicing at these C-terminal sites is thought to have less of an effect upon NMDA receptor pharmacology than N-terminal alternative splicing events.

Expression cloning in *Xenopus* oocytes isolated two different cDNAs encoding functional NMDA receptor subunits. These receptor subunits were termed NMDA-R1A ($NR1_{011}$) and -R1B

($NR1_{111}$) (Durand et al., 1992). The two subunits displayed different pharmacologic properties as a consequence of alternative exon addition within the putative ligand-binding domain. The splicing choice is regulated such that $NR1_{111}$ is the predominate form of the receptor in the cerebellum, whereas $NR1_{011}$ predominates in the cerebral cortex, hippocampus and olfactory bulb. Durand et al. (1992) showed that the functional differences between $NR1_{111}$ and $NR1_{011}$ are marked and include differences in agonist affinity and potentiation by spermine. Clearly, alternative splicing contributes to NMDA receptor diversity. The expression of distinct NMDA receptors with different electrophysiological properties and anatomical distribution may be responsible for the different forms of activity-dependent synaptic plasticity in the mammalian brain such as the generation of different forms of LTP (e.g., hippocampal associative LTP and motorcortical LTP) (Nakanishi et al., 1992).

Recent studies examining the transmembrane topology of a glutamate receptor GluR1 (an AMPA subunit) showed that the N-terminus is extracellular, whereas the C-terminus is intracellular (Hollmann et al., 1994). In addition, three transmembrane domains (TMD), (designated TMD A, TMD B, and TMD C) corresponded to the previously proposed TMDs I, III, and IV, respectively. It was found by N-glycosylation tagging that, contrary to earlier models (Barnard et al., 1987; Hollmann et al., 1989), the putative channel-lining hydrophobic domain TMD II does not span the membrane.

Instead, this domain was suggested to either lie in close proximity to the intracellular face of the plasma membrane or loop into the membrane without traversing it. Furthermore, the region between TMDs III and IV, in previous models thought to be intracellular, is an entirely extracellular domain (Hollmann et al., 1994).

Previously, Durand et al. (1993) showed that the 21-amino acid insert in the N-terminal domain reduced the apparent affinity of homomeric NMDAR1 receptors for NMDA and nearly abolished potentiation by spermine and glycine. For both of these properties, the N-terminal insert was the determining structural feature; the C-terminal domain produced only a very minor effect. These observations correlate with a three transmembrane spanning topology where the N-terminal domain is extracellular and therefore should affect the ligand binding affinity characteristics of the NMDA receptor. Therefore, it is not surprising that the C-terminus would have minimal, if any, effect on ligand binding characteristics due to its intracellular location. This was demonstrated by Durand et al. (1993) when they examined the electrophysiological characteristics of six splice variants of NMDAR1 receptors and concluded that variants differing only in their C-terminal domain showed little change in agonist affinity or spermine potentiation.

The Use of NMDAR1 and NMDAR2A/B Antisera in the Analysis of NMDA Receptor/Channel Protein Density and Distribution in the Rat CNS

One of the first studies examining the NMDAR1 protein in the rat CNS was provided by Hennegriff et al. (1992). Polyclonal antibodies were raised against synthetic peptides corresponding to the carboxyterminal region of the putative NMDA receptor cloned by Nakanishi (1991). The affinity purified antibodies to the NMDA receptor subunit labeled antigens of 75 and 81 kDa. In order to determine the regional distribution of NMDAR1, the 75/81 kDa doublet protein was quantitated by laser densitometry from Western blots in homogenate samples prepared from seven brain regions. They found the doublet to be most abundant in neocortex, followed by hippocampus > striatum >> thalamus > olfactory bulb > cerebellum >> brain stem. The same regional distribution was found in synaptosomes using antibodies to NMDAR1 and resembled earlier autoradiographic studies measuring NMDA-sensitive [^{3}H]glutamate binding sites (Monaghan and Cotman, 1985). A more thorough light and electron microscopic analysis of the distribution of the NMDA receptor subunit NMDAR1 in the rat CNS was performed by Petralia et al. (1994a). This group made a polyclonal antiserum that recognized four of the seven splice variants of NMDAR1 and subsequently utilized this antiserum to perform a comprehensive immunohistochemical survey of the distribution of this antigen. They showed that the NMDAR1

subunit is widespread throughout the rat CNS. The most densely stained cells included the pyramidal and hilar neurons of the CA3 region of the hippocampus, Purkinje cells of the cerebellum and paraventricular neurons of the hypothalamus. Ultrastructural localization of NMDAR1 antigen showed labeling present in postsynaptic densities in a pattern consistent with the synthesis, processing and transport of this protein. Staining was seen in the cytoplasm of dendrites and concentrated in patches associated with groups of microtubules and/or the surface of one pole of a mitochondrion. In addition, patches of staining in the cell bodies were found to form similar associations with microtubules and mitochondria, as well as an association with rough endoplasmic reticulum, Golgi apparatus, and the nuclear envelope. No staining was found in the synaptic cleft. The antiserum did not cross-react with extracts from transfected cells expressing other glutamate subunits, nor did it label non-neuronal tissues. The pattern of staining correlated closely with previous *in situ* hybridization studies but differed somewhat from binding studies (Petralia et al., 1994a).

Another study by this laboratory (Petralia et al., 1994b) examined the histological and ultrastructural localization patterns of the NMDA receptor subunits NMDAR2A and NMDAR2B. They made a polyclonal antiserum to a C-terminus peptide of NMDAR2A. In analysis of membranes from transfected cells, this antiserum recognized NMDAR2A and

NMDAR2B, and to a slight extent, NMDAR2C and NMDAR2D. Immunostained sections of rat brain showed significant labeling throughout the CNS that was similar to that seen previously with their antiserum to NMDAR1. Dense staining was present in postsynaptic densities in the cerebral cortex and hippocampus. Since there is physiological evidence that both NMDAR1 and NMDAR2 subunits coexist in the native NMDA receptor, their findings are consistent with this idea (Petralia et al., 1994b).

Cooperative Modulation of [^{3}H]MK-801 Binding to the NMDA Receptor-ion/Channel Complex by L-Glutamate, Glycine and Polyamines

L-Glutamate binding to the NMDA receptor, in the presence of glycine, produces channel opening. MK-801 is an NMDA receptor antagonist that binds with high affinity (in the low nanomolar range) to a site located within the NMDA channel. L-Glutamate markedly stimulates [^{3}H]MK-801 binding by increasing the affinity for the ligand. Other NMDA receptor agonists (e.g. NMDA, D-aspartate) are capable of enhancing this binding as well, but the non-NMDA receptor agonists (AMPA and kainate) have no effect on [^{3}H]MK-801 binding (Foster and Wong, 1987). Because MK-801 binding has been shown to take place only if the NMDA receptor channel is in the transmitter-activated state (Huettner and Bean, 1988), the efficacy with which L-glutamate produces receptor activation and channel opening can be measured by determining

the amount of [^{3}H]MK-801 binding as a function of glutamate concentration.

Glycine appears to be a constitutive co-agonist for NMDA receptor activation. Glycine alone is ineffective in opening NMDA-linked cation channels. However, glycine greatly potentiates the frequency of channel opening in response to NMDA receptor agonists (Johnson and Ascher, 1987; Reynolds et al., 1987). Glycine also potentiates NMDA-stimulated Ca^{2+} influx in cultured striatal neurons (Reynolds et al., 1987). An allosteric role for glycine in NMDA receptor function is supported by autoradiographic studies. These studies showed that the distribution of strychnine-insensitive [^{3}H]glycine binding sites in supraspinal brain regions mirrored the regional distribution of NMDA-sensitive L-glutamate sites (Monaghan and Cotman, 1985). Since glycine has been shown to promote channel activation in response to agonist, it can be predicted to enhance [^{3}H]MK-801 binding since MK-801 prefers to bind to the activated state of the channel.

Along with the allosteric co-agonist glycine site on the NMDA receptor/complex, there is also a polyamine recognition site. The polyamines spermine and spermidine have been shown to increase both the maximum NMDA response amplitude (Lerma, 1992; McGurk et al., 1990; Ransom and Deschenes, 1990; Sprosen and Woodruff, 1990) as well as the binding of [^{3}H]MK-801 to the NMDA receptor channel (Ransom and Stec, 1988). Because spermine does not activate NMDA receptors in the absence of glutamate and glycine, it has been suggested to

act at an allosteric site independent from the glycine site (Ransom and Stec, 1988).

The mechanism by which L-glutamate and these other agonists affect [^{3}H]MK-801 binding affinity has not been critically addressed. In response to varying agonist concentrations, [^{3}H]MK-801 binding sites may undergo a continuum of conformational changes expressing a smooth range of different affinities for [^{3}H]MK-801. However, it seems more probable that [^{3}H]MK-801 binding is an all (open channel) or nothing (closed channel) phenomenon since most ion channels have not been shown to exhibit broad conductance ranges (i.e. they are either open or closed [Ransom and Stec, 1988]). This would be more consistent with the electrophysiological characterization of MK-801 as a use-dependent, open channel blocker (Foster and Wong, 1987). A more likely explanation for the observed changes in K_D for [^{3}H]MK-801 is that they result from changes in the length of time a channel spends in the activated state (Ransom and Stec, 1988). At high agonist concentrations, channels are opened for a proportionately greater amount of time and the affinity for [^{3}H]MK-801 is high. At low agonist concentrations, the frequency with which the channel opens is comparatively low. The result is an apparent reduction in [^{3}H]MK-801 binding affinity since there is a much lower probability, due to decreased access, that an infrequently opened channel will bind radioligand (Ransom and Stec, 1988).

Specific Aims

The central hypothesis tested in this dissertation project was that there are measurable, anatomically specific aging-related changes in the NMDA receptor/channel complex and its individual subunits. Four specific hypotheses were addressed within the framework of the central hypothesis. The first hypothesis was that there are age-related differences in the density of the NMDA receptor in F-344 rat brain as a function of age. This hypothesis was addressed using *in vitro* quantitative [^{3}H]MK-801 binding analyses (Chapter 3). The second hypothesis was that there are age-related differences in the ability of L-glutamate to enhance [^{3}H]MK-801 binding to NMDA receptors. [^{3}H]MK-801 binding as a function of L-glutamate concentration was performed to test this hypothesis (Chapter 4). The third hypothesis was that there are selective changes in mRNA coding for subunits of the NMDA receptor as a function of age. *In situ* hybridization analyses were performed with cDNA probes specific to NMDA receptor subunits (Chapter 5). The fourth hypothesis was that there are specific age-related changes in NMDAR1 and NMDAR2 protein density in the rat CNS. Immunocytochemistry was performed with antisera specific to the NMDA receptor subunit protein (Chapter 6).

CHAPTER 2
GENERAL METHODS

A description of the general methods that were used throughout this dissertation will follow and will be referred to when describing individual experiments.

Animal Model

Fischer 344 (F-344) male rats 6-, 12-, and 24-months-of-age were used for these experiments. These animals were obtained from the National Institute on Aging (NIA) breeding colony (Harlan). In 1981, the Committee on Animal Models for Research on Aging described the life span characteristics of two rat strains, the F-344 and the Brown Norway (BN) rat, and recommended their use as models for aging research. Among the reasons for choosing F-344 and BN rat strains over other rat strains was that these rats displayed delayed onset of kidney problems (nephropathy) as well as certain tumors. Currently, the NIA recommends and maintains a total of three rat strains for aging studies, the F-344, the BN, and the F_1 F-344/BN hybrid. The F-344 was chosen for these studies because it has long been a standard model for aging research and thus has been extremely well characterized. Survival curves for the F-344 rat show that 24-month-old males have a

40% probability of survival and the probability of survival curve drops rapidly to only 10% by 27-months-of-age. Therefore, 24-month-old animals represent advanced stages of aging in this rat strain and 12-month-old animals can be classified as middle-aged. 6-month-old animals represent young-adults and are used throughout these studies for comparisons to middle-aged and aged rats.

[^{3}H]MK-801 Autoradiography

Tissue Preparation

Male F-344 rats 6-, 12- and 24-months-of-age (n=6 per age group) were decapitated, their brains rapidly removed and frozen with powdered dry ice. Brains were stored at -80 ^{o}C until used. Sections (6μm thick) were cut on a cryostat and representative sections from each age group were thaw-mounted onto chromic acid washed and gelatin-subbed slides. A rat brain atlas (Paxinos and Watson, 1982) was referenced in order to delineate the brain regions for analyses. The brains were sectioned in the horizontal plane. The bregma coordinate corresponded to the dorsoventral distance of the sections from the horizontal plane passing through bregma and lambda on the surface of the skull. The start and stop bregma coordinates were -4.1 mm and -6.1 mm, respectively. This anatomical range was used to thoroughly analyze brain regions of interest that were present on each sectional profile. Every fifth serial section was taken and a total of

fifty sections were cut from each rat brain. One section from each of the three age groups was mounted onto each slide which represented one block of animals. Ten slides were used per block of animals for each [^{3}H]MK-801 binding assay. Two slides per assay were used to determine non-specific binding and these values were subtracted from each total binding value in order to obtain specific binding. These tissue sections were used immediately or stored for no longer than 24 hours at -20 ^{o}C prior to the [^{3}H]MK-801 binding assay.

[^{3}H]MK-801 Binding Assay

Slides were thawed and preincubated at room temperature for 10 minutes in 50 mM Tris acetate with 1.0 mM EDTA and 0.1% saponin (pH 7.7). Sections were then rinsed at 30 ^{o}C for 60 minutes in 50 mM Tris-acetate buffer (pH 7.7). This treatment removes endogenous glutamate, glycine and various ions. Sections were then incubated for 60 minutes at room temperature in 10nM [^{3}H]MK-801 (30 Ci/mmol;New England Nuclear, Boston,MA,U.S.A.) in 50 mM Tris-acetate buffer containing 20 μM D-2-amino-5-phosphonopentanoic acid (D-AP5), 250 μM spermine, 25 μM glycine and 20 μM L-glutamate. D-AP5, a competetive NMDA antagonist, was added to all [^{3}H]MK-801 incubations to give lower and more consistent basal binding levels (Monaghan 1991). The addition of spermine, glycine and L-glutamate ensures a maximal degree of stimulation of the NMDA receptor/ channel complex and therefore optimal [^{3}H]MK-801 binding. Sections were then

washed for 60 minutes in ice-cold Tris-acetate buffer containing 20 μM D-AP5. Nonspecific binding was defined in sections treated identically in the presence of 50 μM MK-801. Following the rinse, the sections were dried under an air stream. The sections were then placed in x-ray cassettes and apposed to tritium-sensitive film (Hyperfilm, Amersham). Tritium standards calibrated against brain paste were included in the cassettes (Microscales, Amersham). The film was exposed for six weeks and then developed using Kodak D-19 developer.

Receptor densities (expressed in pmol/mg protein) from the 12- and 24-month rat brains were normalized against values derived from 6-month-old animals. Data from the 12- and 24-month-old rats were presented as a percentage of the receptor density values from the 6-month-old animals.

Image Analysis

Each slide contained a representative horizontal section from each age group. Ten slides were analyzed per block of animals, with each block consisting of an animal from each age group. Autoradiograms were analyzed, with the investigator blind to age, by computer assisted densitometry with a Microcomputer Imaging Device (MCID, Imaging Research, Inc., St. Catherines, Ont.). Densitometric measurements were converted on line to pmol/mg protein binding.

Statistical Analysis

Six blocks of animals, each block consisting of an animal from each age group, were assessed. The experimental design consisted of a within-subject factor with eight levels (brain region), a between-subject fixed factor with three levels (age), and each of six levels (experimental day). Repeated measures analysis of variance (ANOVA) with between-subject randomized blocks and age considered as a linear covariate were used to model the amount of receptor density as a function of age within each brain region and to determine if this pattern differed significantly among brain regions.

The brain regions that were analyzed included the outer and inner frontal cortices (OFCTX and IFCTX); the entorhinal cortex (ERC); the molecular layer of the dentate gyrus (DG); hippocampal area CA1 stratum radiatum (CA1); the lateral septum (LSEP); the lateral striatum (LSTR); and the lateral thalamus (LTHAL).

Stereological Determination of Neuronal Density

Tissue Preparation

Thirty micron sections of brains from 6-, 12-, and 24-month-old F-344 rats (n=4 per age) were cut on a cryostat. One section from each age group was thaw-mounted on each slide with a total of fifty slides for each block of animals.

The brain regions of interest in these studies were those that showed an age-related decrease in [^{3}H]MK-801 binding as determined by the first experiment in Chapter 3. These regions were the lateral striatum (LSTR), the inner frontal cortex (IFCTX) and the entorhinal cortex (ERC). Sections were stained with Cresyl violet in order to view neuronal cell bodies.

A rat brain atlas (Paxinos and Watson, 1982) was referenced in order to delineate the stereotaxic coordinates of brain regions for analyses. Brains were sectioned in the horizontal plane. Bregma coordinates correspond to the dorsoventral distance of the sections from the horizontal plane passing through bregma and lambda on the surface of the skull. The start and stop bregma coordinates were -3.1 mm and -7.9 mm, respectively. This broader anatomical range was chosen to thoroughly analyze the dorsal to ventral extent of the lateral striatum as well as to view the entorhinal and inner frontal cortices. Two serial sections were taken every 120μm throughout the stereotaxic range.

Data Analysis

Five blocks of animals were used for these analyses with each block containing one 6-month-, one 12-month- and one 24-month-old rat. An initial analysis was performed to ascertain whether the lateral striatum (LSTR) underwent volumetric changes by sampling this brain region through its dorsoventral extent. The volume of this structure could be

readily determined because its entire anatomical distribution could be viewed throughout the series of sections. The LSTR was sampled at five separate anatomical levels (2 serial slides per level) with computer assisted image analysis (Microcomputer Imaging Device, Imaging Research, Inc.). A determination was then made as to whether there was a significant age-related change in the overall volume (mm^3) of this structure. In addition, a quantitative analysis of the laminar thickness of the inner frontal cortex (IFCTX) and the entorhinal cortex (ERC) in 6-, 12- and 24-month-old animals was performed. This study was performed to determine whether there were changes in laminar thickness in these cortical structures in the aged brain when compared to the young animals. Calculations of laminar thickness were used because both the entorhinal cortex and inner frontal cortex have highly organized laminar structures. There were no significant age-related differences in the volume of the LSTR or the laminar thickness of the ERC or the IFCTX.

Neuronal packing density units are in neurons/mm^2. No significant age-related differences in the volume of the LSTR or in the laminar thickness of the ERC or IFCTX were found, therefore packing density should not vary as a function of age. Therefore, any change seen in neuronal packing density would be attributable to differences in the number of neurons per mm^2. In support of this, Coleman et al. (1987) examined the volumes of the components of the hippocampus in the aging F-344 rat and found that there was no change in volume

between 12 and 37 months. They suggested that neuronal packing density (expressed as the number of neurons per mm^2) could be compared in aged animals independent of a volumetric effect.

Determination of Neuronal Density in the Lateral Striatum, Entorhinal Cortex and Inner Frontal Cortex

An MCID impage analysis system was used to determine neuronal packing density in three brain regions. A fully automated sampling scheme was utilized where the general target characteristics were defined. The two target definitions that were used were target density and target size. For the first criteria of target density, MCID was given upper and lower density thresholds (segmentation ranges) and all pixels darker or lighter than the threshold values were ignored. Pixels that were lying within the segmentation range were valid targets. The second criteria for target acceptance was defined as a range of target lengths where the target neurons smaller or larger than the defined size range ($>5\mu m$ and $<20\mu m$) would be ignored with those targets fulfilling the length criteria being counted. After the system found the defined targets, post-scan editing of the digitized image was performed using editing tools. This enabled the investigator to separate any neuronal targets that may have been overlapping.

The area searched for targets (scan area) was also quantified, thus neuronal packing density (number of

neurons/mm^2) was determined for each brain region. Ten serial sections were analyzed from each age group with the investigator blind to age. Neuronal density from 6-month-old animals was used to normalize counts from 12-, and 24-month-old animals. Results were then statistically compared between age groups (one-way repeated measures ANOVA, paired t-tests).

L-Glutamate Stimulation of [^{3}H]MK-801 Binding to NMDA Receptors

Tissue Preparation

Male F-344 rats 6-, 12- and 24-months-of-age (n=6 per age) were decapitated and their brains were rapidly removed and frozen with powdered dry ice. The brains were stored at -80 ^oC until used. Fifty 6μm sections were cut on a cryostat and representative sections from each age group were thaw-mounted onto acid washed and gelatin-subbed slides. These tissue sections were used immediately or stored for no longer than 24 hours at -20 ^oC prior to the [^{3}H]MK-801 binding assay.

[^{3}H]MK-801 Binding Assay

The [^{3}H]MK-801 binding assay was the same as described previously with the exception that varying concentrations of L-glutamate were added to the incubation step in order to examine glutamate's ability to stimulate [^{3}H]MK-801 binding.

The following concentrations (in micromolar) of L-glutamate were used: 0.0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5, 10 and 20. Non-specific binding was defined by the addition of 50 μM unlabeled MK-801 with 20μM L-glutamate. Each slide contained one section from each of the three age groups. A total of four slides, from each block of animals, were analyzed per assay (n=6) for each L-glutamate concentration.

Receptor density (expressed in pmol/mg protein) from 12- and 24-month rat brains was normalized against 6-month-old rats. Data from 12- and 24-month-old animals were statistically compared to receptor density values from 6-month-old animals.

Image Analysis

Each slide consisted of a horizontal section from each age group taken from between bregma coordinates -4.1 mm and -6.1 mm (the dorsoventral distance of the sections from the horizontal plane passing through bregma and lambda on the rat skull). This stereotaxic range was used for a thorough analysis of the brain regions of interest that were present on each sectional profile. A total of fifty sections were cut for each block of animals, with every fifth section being taken throughout the stereotaxic range. Autoradiograms were analyzed, blind to age, by computer assisted densitometry with an MCID system. Four sections from each block of animals, for each concentration of L-glutamate, were analyzed. Two slides per assay were analyzed for non-

specific binding and these values were subtracted from each total binding value in order to obtain specific binding. Densitometric measurements obtained were converted on line to pmol/mg protein binding.

Data Analysis

The brain regions that were analyzed included the OFCTX and IFCTX; the ERC; the molecular layer of the DG; hippocampal area CA1 (stratum radiatum); the LSEP; the LSTR; and the LTHAL.

INPLOT (GraphPad Software, San Diego, CA) was used to generate rectangular hyperbola plots or binding isotherms. These binding isotherm plots yielded EC_{50} (effective concentration at 50%) and E_{max} (maximal effect) values. EC_{50} values and E_{max} values were obtained for each age group and each brain region. Statistical analyses were then performed to ascertain significant age-related differences in these parameters (repeated measures ANOVA; F tests).

In situ Hybridization

Probe Labeling

Oligonucleotide probes for this study were graciously supplied by Dr. Daniel T. Monaghan, University of Nebraska Medical Center, Omaha NE. These probes are 45 nucleotides in length (45 mer) and are specific to NMDAR1, four alternatively spliced versions of NMDAR1 ($NR1_{0xx}$, $NR1_{1xx}$,

$NR1_{x1x}$, $NR1_{xx1}$) and the members of NMDAR2 subunit family (NMDAR2A, NMDAR2B, NMDAR2C, NMDAR2D). The specificity of these probes was previously confirmed by Buller and Monaghan et al. (1994) by incubation of the radiolabeled oligonucleotides in the presence of excess (100 nM) unlabeled probe. In this study, only oligonucleotides with the same sequence inhibited probe hybridization.

The oligonucleotide probes were labeled using a New England Nuclear (NEN) labeling kit and following the methods described by Wisden et al. (1991). The probes were labeled at the 3' end using [^{35}S]dATP (1000-1500 Ci/mmol, NEN) and 3' terminal deoxynucleotidyl transferase (NEN). After labeling, the probes were used immediately or stored for a limited time at -70 ^{o}C.

Tissue Preparation

Frozen brains from 6-, 12- and 24-month-old rats (n=6 per age) were cut at 12 μm, in the horizontal plane, on a cryostat and thaw-mounted on Fisher Superfrost Plus glass slides. The stereotaxic bregma coordinates were from -4.1 mm to -6.1 mm. Sections were refrozen in the cryostat. One section from each age group was mounted per slide for a total of three sections per slide. A total of fifty slides was prepared per block of animals. Four slides per probe, each containing a section from each age group, were used. Gloves were worn at all times during sectioning of the brains and

handling of the slides in order to avoid contamination with ribonucleases.

Fixation

The sections were fixed immediately after cutting by placing the slides in 4% paraformaldehyde at 4°C for 5-15 minutes. Slides were washed in 0.1M phosphate-buffered saline (PBS) for 1 minute and 70% EtOH (RNase-free) for several minutes. Slides were then stored in 95% EtOH in a cold room (4°C) until needed. All solutions were made with diethylprocarbonate (DEPC)-treated water.

Hybridization

Slides were removed from storage and air dried at room temperature. Each labeled probe was dissolved in appropriate amounts of hybridization buffer (NEN, Boston, MA), containing 0.2 M dithiothreitol to achieve a final concentration of 2000 cpm/μl. 100 μl of this solution was applied to the slide and covered with a glass coverslip. Slides were incubated at 42 °C overnight with a parafilm wrap. The following day, parafilm and coverslips were removed and the slides were placed in 1x SSC (saline sodium citrate buffer) at 60 °C for 20 minutes. Finally, the sections were rinsed again in 1x SSC at 60 °C for 5 seconds and then rapidly dried under an air stream.

In situ hybridization is an experimental method that can be used to determine the relative density of individual mRNA

species. Each 45 mer probe was constructed to be highly specific and hybridizes to its particular NMDA subunit mRNA. One potential drawback is that only the relative density of mRNA can be obtained from this method. Therefore, only semi-quantitative analyses were performed. Another drawback is that each probe corresponding to specific NMDA receptor subunits could only be compared across each age group. No quantitative comparisons could be made between various probes. In other words, the relative density of the NMDAR1 mRNA could only be compared between 6-, 12- and 24-month-old animals from the same assay. This information could not be compared to the data obtained for any of the other mRNAs (i.e. NMDAR2A-D and the four alternatively spliced versions of NMDAR1). Another drawback is that the analyses gave an assessment of the relative density of mRNA while not all mRNA is necessarily translated into protein.

Autoradiography

Dried slides were placed in an x-ray cassette and apposed to *B*-max film (Hyperfilm, Amersham) for two weeks, developed in D-19 (Kodak) developer and fixed with Rapid Fix (Kodak). Autoradiograms were analyzed by computer assisted densitometry with a Microcomputer Imaging Device, Imaging Research, Inc.(MCID) image processing system.

Data Analysis

The brain regions analyzed included the OFCTX, IFCTX, LSEP, LSTR, LTHAL, ERC, DG and hippocampal areas CA1 and CA3. Brain regions were compared between the three age groups and the relative density of each subunit mRNA was normalized against the values obtained for young animals. Probes were labeled simultaneously and labeling experiments were grouped such that variability was kept to a minimum. A semi-quantitative measurement of mRNA levels and the distribution of the different subunits was compared between ages.

Immunocytochemistry

Tissue Preparation

Brains from 6-, 12- and 24-month rats (n=5) were serially sectioned at 30 μm on a cryostat and thaw-mounted onto subbed microscope slides and then refrozen in the cryostat. Every fifth section was taken for a total of fifty slides. One brain section from each block of animals was mounted per slide n=5.

Antibody Specificity

AB1516 (Chemicon Intl. Inc., Temecula, CA), an antiserum raised against a synthetic peptide corresponding to the C-terminus of rat NMDA receptor subunit (NMDAR1), is selective for splice variants $NR1_{011}$, $NR1_{111}$, $NR1_{001}$, $NR1_{101}$. These

appear to be the major splice variants expressed in rat brain (Hollmann et al., 1993). It has been shown previously that there is no cross reactivity of this antiserum with other glutamate receptor subunits (Petralia et al., 1994a). AB1548 (Chemicon Intl. Inc., Temecula, CA), an antiserum raised against a synthetic peptide corresponding to the C-terminus of rat NMDAR2A receptor subunit, recognizes both NMDAR2A and NMDAR2B subunits equally. This antiserum shows no cross reactivity with NMDAR1 or other glutamate receptor subunits (Petralia et al., 1994b).

Immunocytochemical Procedure

An optimal antibody concentration was determined empirically. Previous studies had used an AB1516 antibody concentration between 2 and 4 μg/ml (Petralia et al., 1994a) and AB1548 antibody concentration of 0.5-1.5 μg/ml (Petralia et al., 1994b). In the present experiments, a concentration of 2.5 μg/ml was found to produce optimal results with both NMDAR1 and NMDAR2A/B antibodies. Sections were incubated in 10% normal goat serum in PBS (pH 7.4) for 1 hour and then kept overnight in primary antisera (AB1516 or AB1548) in PBS. Sections were then washed and incubated in biotinylated goat anti-rabbit antisera (1:250 dilution) for 1 hour, washed, incubated in avidin-horse radish-peroxidase (1:200 dilution) for 1 hour, washed, treated for 15 minutes with 3',3-diaminobenzidine tetrahydrochloride (0.5 mg/ml PBS + 5 μl/ml of 0.6% hydrogen peroxide), and washed. All washes were

performed with PBS (2 x 15 min.). Sections with PBS substituted for the primary antibody (PBS controls) were also used in order to correct for nonspecific staining.

Staining density was semi-quantitated by an MCID image analysis system. Data were presented as raw optical densities. Raw optical density of NMDAR1 and NMDAR2A/B immunoreactivity from 6-month-old animals was used to normalize data from 12- and 24-month-old animals. Data from middle-aged and aged animals were presented as a percentage of the six-month-old animals.

CHAPTER 3
AGE-RELATED CHANGES IN [^{3}H]MK-801 BINDING IN F-344 RATS

Introduction

Aging is associated with a reduction in many physiologic forms of neuronal plasticity. Recent findings indicate a direct correlation in age-dependent deficits in spatial memory and in hippocampal kindling (a measure of neuronal plasticity) as measured in 26-month-old Fischer 344 rats (DeToledo-Morrell et al., 1984). N-Methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmission has been shown to be important for learning and memory in animals and man (Collingridge, 1987; Cotman and Iversen, 1987; Harris, 1984). Profound learning and memory impairments are seen in a variety of animal species after NMDA-receptor activation is blocked by either competitive or non-competitive antagonists (Morris et al., 1986). There are also some striking similarities between NMDA antagonist induced memory impairments and age-related cognitive deficits, suggesting that age-related alterations in NMDA receptors may be involved in cognitive decline with increasing age (Bonhaus et al., 1990; Ingram et al., 1992).

To test the hypothesis that NMDA receptors decline during aging, an examination of the density of NMDA receptors was performed in aged Fischer 344 rats. In this study, [^{3}H](+)-5-methyl-10,11-dihydro-5H-dibenzo(a,d)-cycloheptan-5,10-iminehydrogen maleate (MK-801 or dizocilpine), a highly specific non-competitive NMDA receptor antagonist was used to determine the density of NMDA receptors. This antagonist binds with high affinity (low nanomolar range K_D) to a site located within the NMDA receptor ion channel. Thus, MK-801 binding occurs only if the NMDA receptor is in the transmitter-activated (open) state (Huettner and Bean, 1988). Channel activation by an NMDA receptor agonist like L-glutamate, in the presence of glycine, promotes high-affinity MK-801 binding. Glycine alone cannot open NMDA receptor channels, but is a constitutive co-agonist. Glycine, in the presence of L-glutamate or other NMDA receptor agonists (i.e., NMDA or D-aspartate) has been shown to greatly potentiate the frequency of channel opening (Johnson and Ascher, 1987; Reynolds et al., 1987). In addition, the polyamines spermine and spermidine increase both the maximum NMDA response amplitude as well as binding of MK-801 to the NMDA receptor. Alone, polyamines do not activate the NMDA receptor, suggesting the presence of an allosteric binding site independent from the other co-agonist sites (Lerma, 1992; McGurk et al., 1990; Ransom and Deschenes, 1990; Sprosen and Woodruff, 1990).

Because of the high degree of specificity and affinity of MK-801 for the NMDA receptor channel complex, [^{3}H]MK-801 is an ideal ligand to examine age-related changes in NMDA receptors in the CNS. Also, because MK-801 only binds to the activated state of the NMDA receptor, the total number of available NMDA receptors can be determined by using optimal agonist and co-agonist concentrations.

Methods

For a detailed description of the experimental tissue used in these studies, the [^{3}H]MK-801 binding assay procedure, and image analyses see Chapter 2.

Statistical Analysis for [^{3}H]MK-801 Binding

The data acquired in this study consisted of quantitative analyses of the average density of [^{3}H]MK-801 binding (pmol/mg protein) obtained from bilateral brain regions for each of the 6-,12-,and 24-month-old rats (n=6 for each age). One "group" consisted of one 6-month-, one 12-month-, and one 24-month-old animal mounted per series of slides. Ten slides, from each of these groups, were analyzed per assay. The regions that were analyzed included the outer frontal cortex (OFCTX), inner frontal cortex (IFCTX), entorhinal cortex (ERC), dentate gyrus of the hippocampus (DG), hippocampal area CA1, lateral septum (LSEP), lateral striatum (LSTR) and the lateral thalamus (LTHAL).

Repeated measures analysis of variance (ANOVA) with between-animal randomized blocks and age considered as a linear continuous covariate was used to model the pattern of MK-801 binding as a linear function of age within each brain region. F-tests were also performed for the presence of a significant interaction between age and brain region receptor density effects. In assessing the validity of ANOVA assumptions, it was noted that experimental day age profiles of receptor binding were parallel within each brain region and that between-animal variability as estimated by the standard deviation was similar among age groups within each brain region. This indicated that the patterns of [^{3}H]MK-801 binding obtained for each group of 6-, 12-, and 24-month-old rats were the same, independent of which experimental day each group was assayed. Within age groups however, brain region between-animal standard deviations tended to increase as the mean level of receptor density in those regions increased. ANOVA models assume that standard deviations do not vary in this manner. Therefore, receptor binding density values were transformed logarithmically, prior to analysis, in order to appropriately model this inherent pattern of variability within the ANOVA framework. Plots of residuals versus predicted values, residual histograms, and residual normal probability plots were examined to assess goodness of fit in the ANOVA model. The statistical analyses determined which mean age profiles of MK-801 binding density appeared to

be linearly decreasing in specific brain regions and which profiles had slopes which were significantly less than zero.

Results

[^{3}H]MK-801 Binding

The IFCTX, ERC and LSTR were the only regions analyzed that underwent a significant change (decrease) in [^{3}H]MK-801 binding when 24-month-old animals were compared to 6-month-old animals. The mean age profiles for the IFCTX, ERC and the IFCTX all linearly decreased and had slopes significantly less than 0 with p values of .0026, .0151 and .0018 respectively (Figure 3-1). The other brain regions analyzed, the OFCTX, the LSEP, the LTHAL, the molecular layer of the DG and hippocampal area CA1, did not change as a function of increasing age (Figures 3-2 and 3-3). A representative autoradiograph shows the distribution of [^{3}H]MK-801 binding in the rat brain (Figure 3-4).

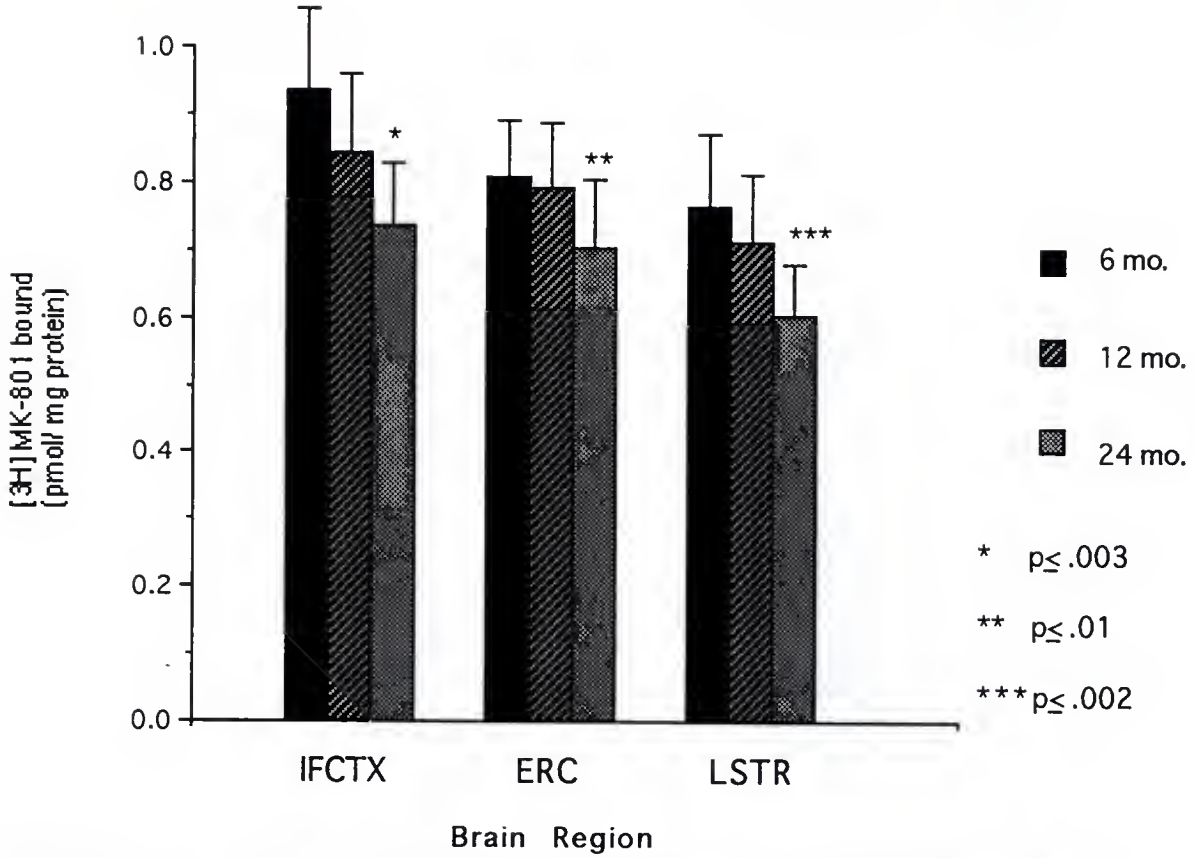


Figure 3-1. Brain regions that underwent a significant age-related decrease in [^{3}H]MK-801 binding. (IFCTX=inner frontal cortex; ERC=entorhinal cortex; LSTR=lateral striatum). Repeated measures ANOVA with between-animal randomized blocks and age as a linear covariate presented as a function of increasing age. The p values represent mean age profiles with slopes significantly less than 0.

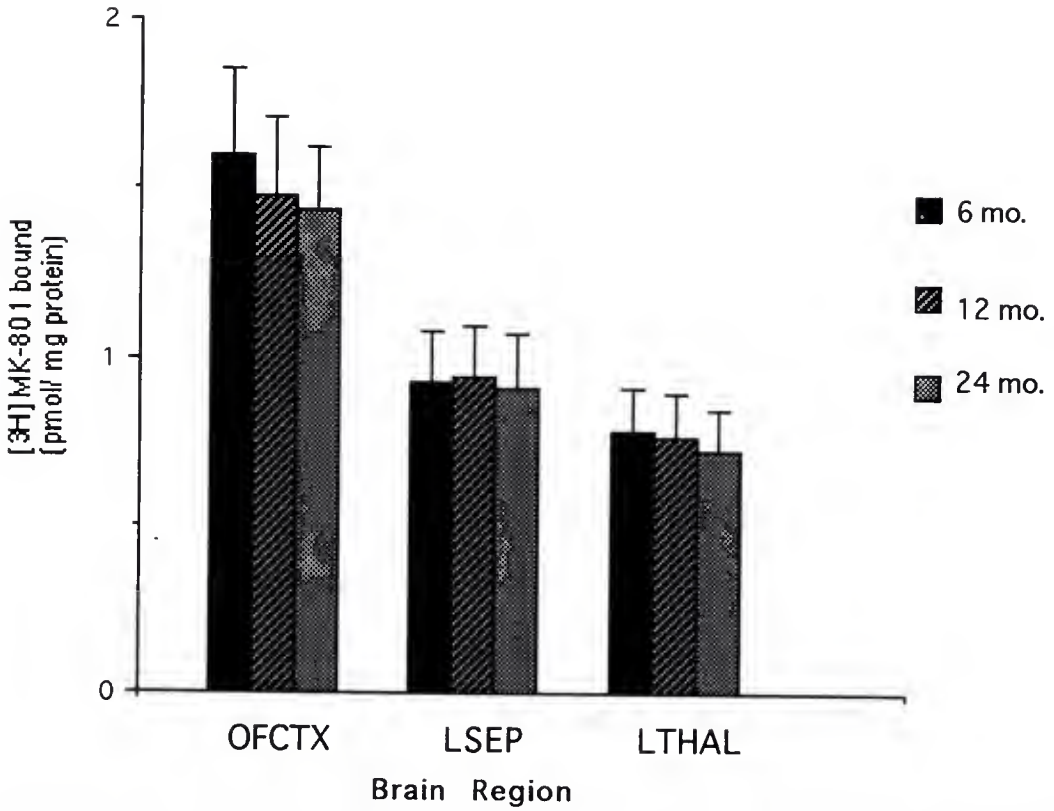


Figure 3-2. Brain regions that did not undergo a significant age-related change in [^{3}H]MK-801 binding. (OFCTX= outer frontal cortex; LSEP= lateral septum; LTHAL= lateral thalamus). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate presented as a function of increasing age.

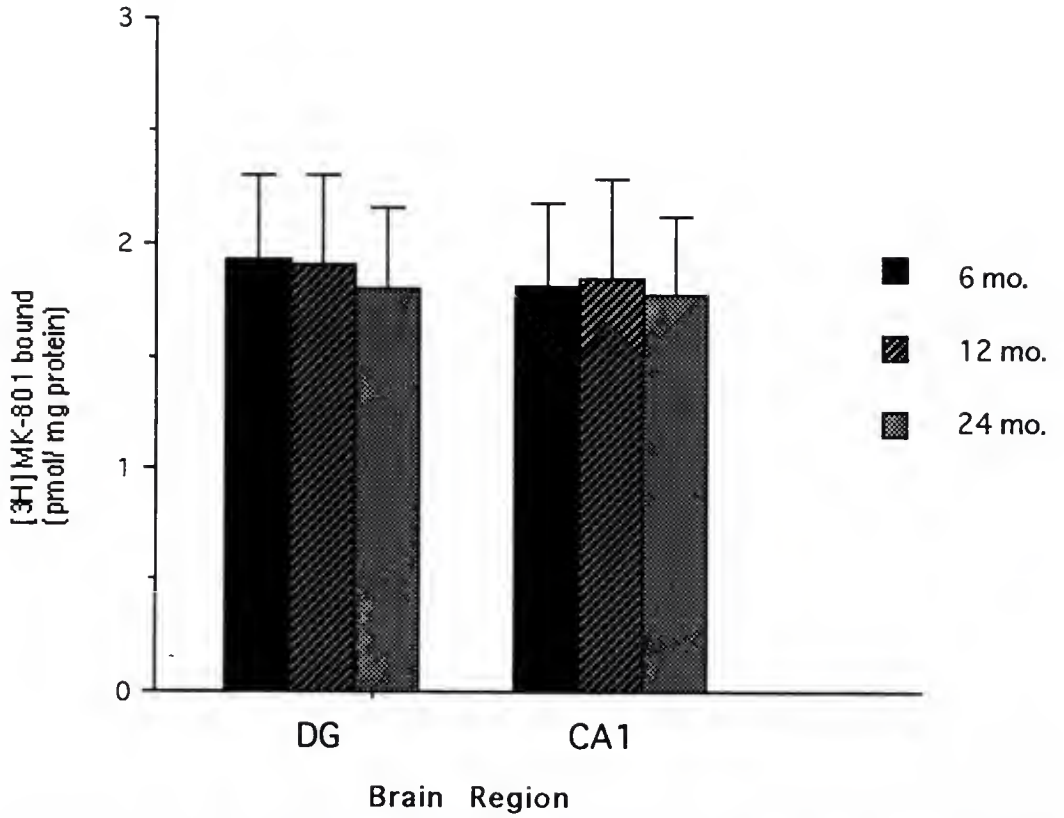


Figure 3-3. Brain regions that did not undergo a significant age-related change in [^{3}H]MK-801 binding. (DG= molecular layer of the hippocampal dentate gyrus; CA1= hippocampal area CA1 stratum radiatum). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate presented as a function of increasing age.

Figure 3-4. NMDA receptor distribution as determined by [^{3}H]MK-801 binding in : (A) 6-month-old, (B) 12-month-old, and (C) 24-month-old rat brain.

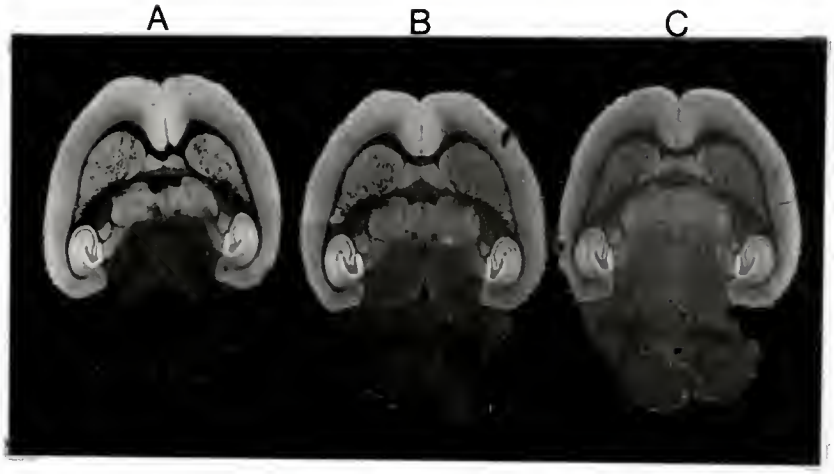

A
B
C

TABLE 3-1. Laminar thickness (mm), volume (mm^3), and neuronal density (neurons/mm^2) values from Fischer 344 rat brain.

	6-month-old	12-month-old	24-month-old
Laminar Thickness (mm)			
Frontal Cortex	Mean= 0.1952 SEM = 0.0013	0.19561 0.00211	0.1945 0.0017
Entorhinal Cortex	Mean= 0.2988 SEM = 0.0031	0.2940 0.0037	0.2980 0.0026
Volume (mm^3)			
Lateral Striatum	Mean=41.8491 SEM = 1.2064	41.8147 1.0065	41.3812 0.5626
Neuronal Density (per mm^2)			
Entorhinal Cortex	Mean=1343.86 SEM = 36.60	1300.38 127.03	1319.50 41.33
Inner Frontal Cortex	Mean=1285.50 SEM = 35.17	1279.89 10.86	1285.31 33.81
Lateral Striatum	Mean=1284.23 SEM = 24.01	1284.71 51.28	1297.88 21.01
n=5 animals per age group			

No statistically significant differences found with ANOVA (multi-comparison significance level at 95%, repeated measures).

Discussion

These studies showed that the number of NMDA receptors, as determined by [^{3}H]MK-801 binding, is reduced in the ERC, the LSTR and the IFCTX in aged F-344 rats. However, receptor density in the OFCTX, the LSEP, the LTHAL, the molecular layer of the DG as well as hippocampal area CA1 did not change as a function of increasing age.

It is interesting that the decrease in the density of [^{3}H]MK-801 binding was limited to only three of the eight CNS structures analyzed. The ERC was one structure that did undergo an age-related decrease in MK-801 binding. The ERC projects to the hippocampal formation, a component of the limbic system associated with brain mechanisms for memory. More specifically, the ERC is the major source of extrinsic afferents to the dentate gyrus granule cells and pyramidal cells of the hippocampus proper (Amaral and Witter, 1989). There are decreased numbers of NMDA receptors in the ERC of aged rats and this may affect the connectivity from this structure to the hippocampus. This may result in an alteration in NMDA receptor-mediated neurotransmission and subsequently alter some aspects of memory (e.g., spatial information processing) (Barnes and McNaughton, 1985; Morris et al., 1978). It has been shown that excitotoxic lesions of the rat ERC impair the retention of reference memory tasks (Levisohn and Isacson, 1991). Age-dependent deficits in spatial memory have been shown to be related to impairments

in hippocampal "kindling" (used as a measure of neuronal plasticity) in F-344 rats (DeToledo-Morrell et al., 1984). The kindling phenomenon is dependent upon sequential synaptic modification in a cascading neural system (DeToledo-Morrell et al., 1984), and would be expected to be especially vulnerable to age-related alterations in postsynaptic mechanisms such as a decrease in NMDA receptors in the entorhinal cortex. The ERC, in addition to having connections with the hippocampus, has reciprocal connections with a wide range of cortical association areas (Amaral and Witter, 1989). The decrease in MK-801 binding in the ERC could therefore also affect cortical circuitry by decreasing the overall amount of NMDA receptor-mediated neurotransmission.

The decrease in MK-801 binding observed in the IFCTX of the aged animals may contribute to some alteration in the circuitry necessary for learning and memory as well. Cortical-striatal projections have been shown to utilize NMDA receptors (Cherubini et al., 1988), and a decreased density of NMDA receptors in both IFCTX and the LSTR may affect the NMDA receptor-mediated neurotransmission between these projections. Piggott et al. (1992) found that [^{3}H]MK-801 binding declined with age in human frontal cortical membranes. More recently, Serra et al. (1994) found that the total number of binding sites for [^{3}H]MK-801 was decreased in the hippocampus, cerebral cortex and striatum of 18- and 24-month-old rats, relative to 3-month-old animals. Other

groups have also shown a significant age-related decrease in [^{3}H]MK-801 binding in cerebral cortical and hippocampal brain homogenates (Kitamura et al., 1992; Tamaru et al., 1991). In another study, the ERC, IFCTX and LSTR were among various brain regions that were shown to undergo the greatest percent decline in NMDA-displaceable [^{3}H]L-glutamate binding when 30-month-old mice were compared to 3-month-olds (Magnusson and Cotman, 1993). These regions may be some of the most vulnerable to the effects of aging. Since these regions are implicated in normal memory function and plasticity of the CNS (DeToledo-Morrell et al., 1984; Gonzales et al., 1991; Petit, 1988), an age-related decrease in NMDA receptors in these regions suggests that some of the cellular mechanisms encoding memory are subsequently impaired.

It was determined that the age-related decrease seen in [^{3}H]MK-801 binding was not a result of a decrease in neuronal density in the ERC, IFCTX or the LSTR. Age comparisons were made in neuronal packing densities for each of these brain regions. As West et al. (1993) stated, the volume and/or potential shrinkage (laminar thickness) of specific brain regions needs to be determined in order to obtain accurate measurements of changes that may be occurring in specific neuronal populations. He stated that only when these parameters are known can neuron packing density be used, instead of total neuronal number, in a meaningful discussion of the functional state of a neural structure (West et al, 1993). In the present study, neuronal packing density was

measured as the number of neurons per square millimeter. Therefore, this measurement would only be valid after determining that these brain regions did not undergo an age-related change in overall volume (LSTR) or laminar thickness (ERC and IFCTX) (i.e., the mm^2 denominator value was not affected by age). No significant age-dependent differences were found in the volume of the LSTR or in the laminar thickness of the ERC and IFCTX (Table 3-1). Neuronal packing densities were then determined utilizing an MCID image analysis system for the ERC, the IFCTX and the LSTR. There was no statistically significant difference in neuronal density measurements in any brain region examined. Therefore the decrease seen in [^{3}H]MK-801 binding represents a loss of NMDA receptors within these brain regions rather than a loss of neurons.

In this study, [^{3}H]MK-801 binding density was expressed in pmol/mg protein. Therefore, changes in protein concentration in specific brain regions as a function of age may effect these values. Burnett and Zahniser (1989) performed a quantitative autoradiographic study that examined age-related changes in α-1 adrenergic receptors in F-344 rat brain. Protein levels were measured in the same tissue sections by using a staining procedure described by Miller et al. (1988). They found no significant differences in protein concentration between age groups (i.e., 3- to 4-month-old, 16- to 18-month-old and 24- to 28-month-old) in the thalamus, cerebral cortex, hippocampus, striatum, cerebellum,

brainstem, and olfactory tubercle. Since the ages and strain of rats used in the present study were identical to Burnett and Zahniser (1989) it is expected that total protein would also remain unchanged as a function of age. Therefore, it is probable that age-related differences in [^{3}H]MK-801 binding density in specific brain regions is due to alterations in receptor number and not in protein concentration.

It should be noted that finding an age-related effect on [^{3}H]MK-801 binding in only three of eight brain regions analyzed may reflect the inability of MK-801 to label all of the NMDA receptors, since ligand binding can only assay surface-presented receptors. Studies investigating mRNA coding for the NMDA receptor is presented in Chapter 5. The density of NMDA protein is also presented in Chapter 6. These further studies address the hypotheses that the density of mRNA coding for the NMDA receptor and the density of NMDA protein change as a function of age.

These [^{3}H]MK-801 binding experiments have determined some of the age-dependent changes taking place in the NMDA receptor/channel complex. The decrease in NMDA receptor density seen in specific brain regions may account for some of the deleterious effects of aging on learning and memory (Barnes, 1979; Barnes and McNaughton, 1985). Gonzales et al. (1991) studied NMDA receptor-mediated responses in the hippocampus, cortex, and striatum of F-344 rats of various ages (3- to 5-, 12- to 14-, and 24- to 28-months-of-age) to determine whether aging alters some of the functional

properties of this receptor complex. They examined NMDA-stimulated release of norepinephrine and dopamine as indices of NMDA receptor function and found that NMDA-mediated responses were attenuated with increasing age. In Chapter 4, the effects of increasing age on L-glutamate's ability to enhance [^{3}H]MK-801 binding is studied. This approach was taken to see if there were differences in the E_{max} (maximal response or density of [^{3}H]MK-801 bound) or EC_{50} (the concentration of L-glutamate producing a half-maximal response) as a function of age.

Many of the processes underlying learning and memory are known to utilize, to a great extent, NMDA receptors (Danysz et al., 1988; Morris et al., 1986). Therefore, elucidation of age-dependent changes in NMDA receptors may help target potential pharmaceutical interventions aimed at alleviating age-related cognitive decline. It has been suggested that pharmacological manipulation of glutamatergic neurotransmission may prove beneficial for cognitive enhancement (Ingram et al., 1994). However, the utilization of NMDA receptor agonists may be an unsafe strategy due to the potential neurotoxic effects associated with overstimulation of the NMDA receptor (Cotman and Monaghan, 1988). Therefore, indirect activation through other modulatory sites identified on the NMDA receptor complex could be investigated, such as the co-agonist glycine site and/or the polyamine modulatory sites.

Milacemide and D-cycloserine are two strong candidates for agonists at the glycine site and have both been shown to enhance memory performance in rats (Flood et al., 1992; Quartermain et al., 1991). A relatively new class of chemical substances, nootropics, were developed specifically to alleviate age-related cognitive deficits and have been shown to enhance NMDA receptor density (10 to 25%) in aged brain (Cohen and Muller, 1992; Davis et al., 1993; Fiore and Rampello, 1989). Phosphatidylserine is one example of a nootropic drug. Cohen and Muller (1992) showed that chronic treatment with phosphatidylserine ameliorated age-associated deficits of the NMDA receptor in the forebrain of aged mice by restoring the density of NMDA receptors to levels similar to young animals and also by normalizing the sensitivity of aged animals for the stimulating effects of L-glutamate and glycine. Cohen and Muller (1993) also showed significant enhancement (20%) of NMDA receptor density in aged mice treated with another nootropic drug, piracetam. The kinetic constants for L-glutamate stimulation of [^{3}H]MK-801 binding in the aged animals were not significantly different from untreated young mice (Cohen and Muller, 1993). In addition, the nootropic agent L-acetylcarnitine was shown to attenuate the age-dependent decrease of NMDA-sensitive glutamate receptors in the rat hippocampus (Davis et al., 1993; Fiore and Rampello, 1989). Taken together, these studies imply that specific pharmacological targeting of the NMDA receptor

may indeed prove to be a viable treatment strategy for restoration of cognitive deficits in the aged population.

CHAPTER 4
EFFECT OF AGE ON L-GLUTAMATE STIMULATION OF [^{3}H]MK-801 BINDING IN F-344 RAT BRAIN

Introduction

In Chapter 3, a decrease in the number of NMDA receptors in specific brain regions in the F-344 rat was shown. Other studies have described a similar reduction in NMDA receptors in various species (Anderson et al., 1989; Ingram et al., 1992; Magnusson and Cotman, 1993; Peterson and Cotman, 1989; Serra et al., 1994; Tamaru et al., 1991; Wenk et al., 1991) including humans (Piggott et al., 1992). In Chapter 3, [^{3}H]MK-801 was utilized to study the NMDA receptor/channel complex. This antagonist binds with high affinity to a site within the channel of the NMDA receptor complex, thus MK-801 binding will only take place if the NMDA receptor is in the transmitter-activated (open) state (Huettner and Bean, 1988). Channel activation by an NMDA agonist such as L-glutamate, in the presence of glycine allows MK-801 binding to take place. Glycine alone cannot activate the NMDA receptor. In addition to the glycine site on the NMDA receptor/complex, there is an allosteric polyamine recognition site. The polyamines spermine and spermidine have been shown to increase both maximum NMDA response amplitude (Lerma, 1992; McGurk et al.,

1990; Ransom and Deschenes, 1990; Sprosen and Woodruff, 1990) as well as binding of MK-801 to the NMDA receptor channel (Ransom and Stec, 1988).

L-glutamate is the endogenous transmitter for the NMDA receptor channel complex. In this study, an evaluation of the efficacy of L-glutamate to stimulate MK-801 binding within the NMDA receptor/channel complex was performed by using various concentrations of L-glutamate. These studies examined the efficacy and potency with which L-glutamate produces receptor activation and channel opening as a function of increasing age by comparing dose-response curves from 6-, 12-, and 24-month-old animals.

The hypothesis tested in these experiments was that there would be an age-related decrease in the maximal response (E_{max}) elicited by L-glutamate. It was also hypothesized that there would not be a concomitant change in the concentration of L-glutamate producing a half-maximal response (EC_{50}) as a function of age. In these experiments, the response of the NMDA receptor was defined as the density of [^{3}H]MK-801 binding determined as a function of L-glutamate concentration.

Many studies have shown that there are decreased numbers of NMDA receptors in aged animals (Anderson et al., 1989; Ingram et al., 1992; Magnusson and Cotman, 1993; Peterson and Cotman, 1989; Piggott et al., 1992; Serra et al., 1994; Tamaru et al.,1991; Wenk et al., 1991). Consequently, there are fewer NMDA receptors available for occupation by ligand

in aged rats. Because there are fewer NMDA receptors available for occupation by ligand in aged rats it would be expected that there would be a decreased maximal response ([^{3}H]MK-801 bound) in aged rats due to fewer NMDA receptor channels available to be activated (opened) by agonist. In addition, previous studies have shown an age-related decrease in the functional capacity of the NMDA receptor/channel complex as defined by a reduction in various NMDA-mediated responses (Gonzales et al., 1991; Pittaluga et al., 1993; Serra et al., 1994). The ability of L-glutamate to stimulate [^{3}H]MK-801 binding was therefore compared across varying age groups in the F-344 rat.

Methods

Tissue Preparation

The male F-344 rats, 6-, 12- and 24-months-of-age, that were used in these experiments were the same as those described in Chapter 3. For a more detailed description of the tissue preparation see Chapter 2.

[^{3}H]MK-801 Binding Assay

For a detailed description of this protocol, see Chapter 2. Varying concentrations of L-glutamate (i.e., 0.0 μM, 0.05 μM, 0.1 μM, 0.25 μM, 0.5 μM, 1.0 μM, 2.5 μM, 5.0 μM, 10 μM, and 20 μM) were added to the incubation step in order to examine glutamate's ability to stimulate [^{3}H]MK-801 binding.

A total of four slides, from each block of animals, were analyzed per assay (n=6) for each L-glutamate concentration.

Image analysis

See Chapter 2 for a detailed description of image analysis.

Data Analysis

The data consist of the EC_{50} and E_{max} values obtained from binding isotherm plots. These plots were generated from average density measurements of [^{3}H]MK-801 binding (pmol/mg protein) in the presence of increasing concentrations of L-glutamate. Binding densities were obtained from bilateral brain regions for each of the 6-,12-, and 24-month-old rats (n=6 for each age). One "group" consisted of one 6-month-, one 12-month-, and one 24-month-old animal mounted per series of slides. Each [^{3}H]MK-801 binding assay was run with the three ages of animals mounted per slide. Four slides, from each of these groups, were analyzed per assay per L-glutamate concentration, with each [^{3}H]MK-801 binding assay being performed on one group of animals (n=6). Two slides per assay were analyzed for non-specific binding and these values were subtracted from each specific binding value in order to obtain total binding. Brain regions that were analyzed included the outer and inner frontal cortices (OFCTX, IFCTX); the entorhinal cortex (ERC); the molecular layer of the dentate gyrus (DG); hippocampal area CA1 stratum radiatum

(CA1); the lateral septum (LSEP); the lateral striatum (LSTR); and the lateral thalamus (LTHAL).

Binding Isotherm Plots

INPLOT (GraphPad Software, San Diego, CA) was used to generate linear regression plots from the density values obtained in these experiments. Plots gave estimated EC_{50} (effective concentration at 50%) and E_{max} (maximal [^{3}H]MK-801 binding elicited by L-glutamate) values for L-glutamate stimulation of [^{3}H]MK-801 binding. Estimates were then used to generate rectangular hyperbola plots or binding isotherms. Binding isotherm plots then yielded the true EC_{50} and E_{max} values for each age group and brain region analyzed. As the concentration of L-glutamate increased, there was a concominant increase in the density of [^{3}H]MK-801 binding (Figure 4-1). EC_{50} and E_{max} values obtained from [^{3}H]MK-801 binding isotherm plots for the 6-month-old animals were used to normalize the 12- and 24-month-old animal values.

Statistical Analysis

Six blocks of animals, each block consisting of an animal from each age group, were assessed. A different block of 3 animals, 1 per age group, was assessed on each of 6 experimental days. The experimental design consisted of a within-animal factor with 8 levels (brain region), a quantitative between-animal fixed factor with 3 levels (age), and a between-animal random blocking factor with 6 levels

(experimental day). Repeated measures analysis of variance (ANOVA) with between-animal randomized blocks and age considered as a linear covariate were used to model the EC_{50} and E_{max} data as a linear function of age within each brain region and to determine if this pattern differed significantly among brain regions. The change in EC_{50} and E_{max} per month-of-age (i.e. the linear slope) was estimated and assessed for statistical significance within each brain region while blocking on experimental day. A general comparison of these slopes among brain regions was then performed by testing for the presence of a significant interaction between age and brain region EC_{50} and E_{max} effects. Mauchly's sphericity test was used to determine if a multivariate F test (Wilks' Lambda) or a univariate F test should be used for the age x brain region interaction test. In assessing the validity of ANOVA assumptions, it was noted that experimental day age profiles were relatively parallel within each brain region and that between-subject variability as estimated by the standard deviation was similar among age groups within each brain region. Plots of residuals versus predicted values, residual histograms, and residual normal probability plots were examined to assess goodness of fit in the ANOVA models.

Results

[^{3}H]MK-801 binding increased as a function of L-glutamate concentration in all the age groups and a

representative autoradiogram is presented in Figure 4-1. Significant differences between the E_{max} values in nearly all brain regions were found when middle-aged and aged animals were compared to the young rats (Table 4-1). The only region where E_{max} did not differ in middle-aged animals, when compared to young-adults, was the OFCTX. The areas showing the greatest percent decline in aged animals when compared to young were the LSTR (26.4%), ERC (24.7%), and IFCTX (21.5%). Areas showing the greatest percent decline in middle-aged animals when compared to young were the LSTR (12.9%), LSEP (10.3%), and IFCTX (9.3%).

No significant differences were found in the EC_{50} values when comparing middle-aged and aged rats to young (Table 4-2).

TABLE 4-1. E_{max} values (pmol/mg protein) from [^{3}H]MK-801 binding isotherms.

BRAIN REGION	6 month	12 month	24 month
OFCTX	1.588 ± 0.29	1.468 ± 0.29	1.441 ± 0.24*
IFCTX	0.959 ± 0.16	0.870 ± 0.17*	0.753 ± 0.14*
ERC	0.830 ± 0.12	0.758 ± 0.14*	0.625 ± 0.12*
DG	1.874 ± 0.37	1.737 ± 0.40*	1.669 ± 0.39*
CA1	1.712 ± 0.36	1.618 ± 0.41*	1.537 ± 0.40*
LSEP	0.951 ± 0.20	0.853 ± 0.19*	0.850 ± 0.21*
LSTR	0.780 ± 0.15	0.679 ± 0.14*	0.574 ± 0.12*
LTHAL	0.793 + 0.15	0.747 + 0.15*	0.665 + 0.14*
	MEAN ± SEM	MEAN ± SEM	MEAN ± SEM
	n=6	n=6	n=6

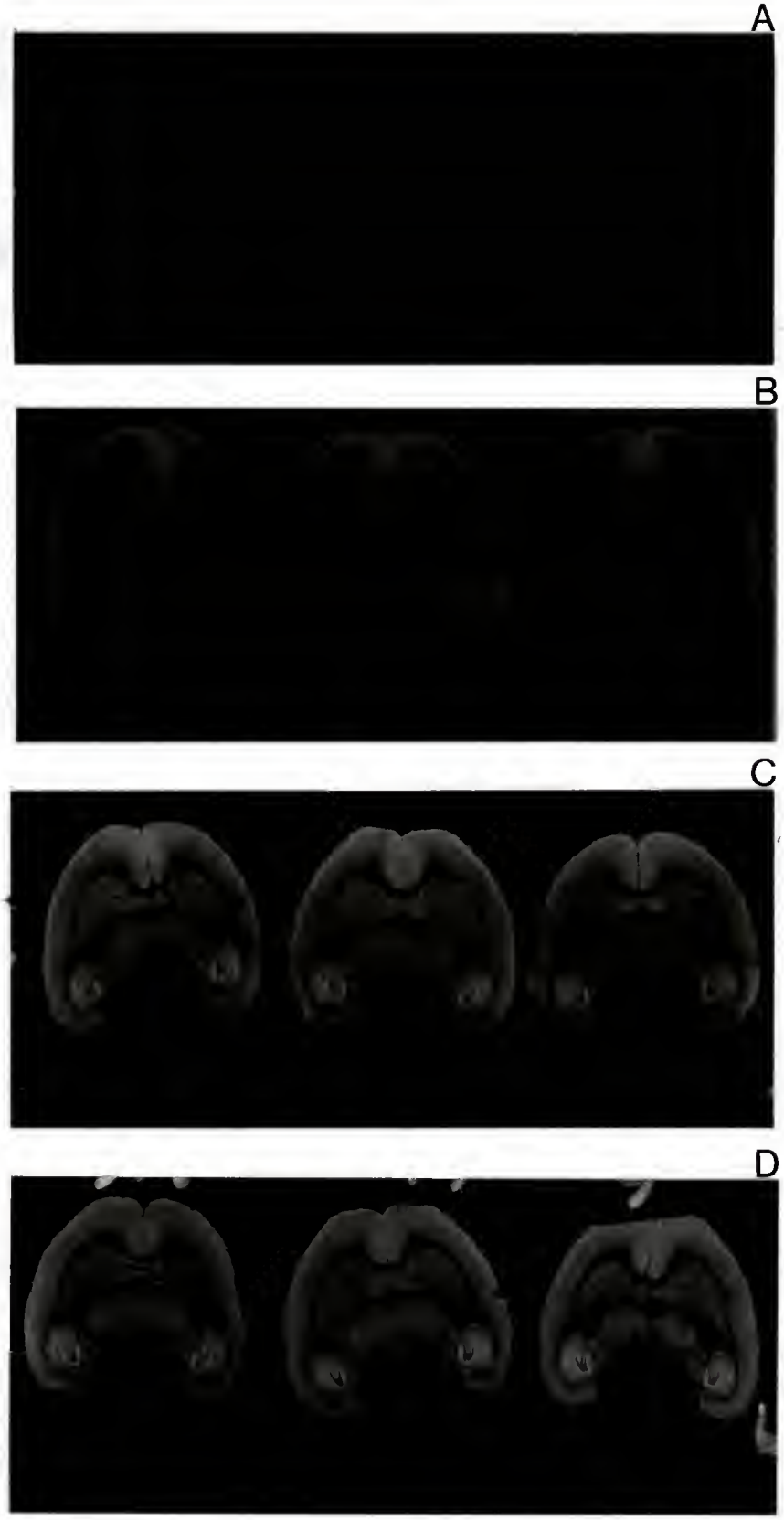
Asterisks (*) indicates significance when compared to 6-month ($p \leq 0.05$ ANOVA; F-test). Abbreviations: OFCTX=outer frontal cortex; IFCTX=inner frontal cortex; ERC=entorhinal cortex; DG=molecular layer of the dentate gyrus; CA1=hippocampal area CA1; LSEP=lateral septum; LSTR=lateral striatum; LTHAL=lateral thalamus

TABLE 4-2. EC_{50} values (μM) from [^{3}H]MK-801 binding isotherms.

BRAIN REGION	6 month	12 month	24 month
OFCTX	0.764 ± 0.14	0.758 ± 0.18	0.798 ± 0.22
IFCTX	1.311 ± 0.23	1.085 ± 0.26	1.148 ± 0.39
ERC	1.108 ± 0.17	1.197 ± 0.32	0.921 ± 0.25
DG	0.951 ± 0.21	0.936 ± 0.25	1.040 ± 0.20
CA1	0.782 ± 0.17	0.660 ± 0.17	0.725 ± 0.20
LSEP	0.432 ± 0.11	0.444 ± 0.14	0.410 ± 0.14
LSTR	0.578 ± 0.13	0.586 ± 0.15	0.599 ± 0.23
LTHAL	0.993 + 0.20	0.861 + 0.18	0.892 + 0.23
	MEAN ± SEM	MEAN ± SEM	MEAN ± SEM
	n=6	n=6	n=6

No significant differences were found when 12-, and 24-month-old rats were compared to 6-month-old rats ($p \geq 0.05$ ANOVA; F-test). Abbreviations: OFCTX=outer frontal cortex; IFCTX=inner frontal cortex; ERC=entorhinal cortex; DG=molecular layer of the dentate gyrus; CA1=hippocampal area CA1; LSEP=lateral septum; LSTR=lateral striatum; LTHAL=lateral thalamus

Figure 4-1. Total [^{3}H]MK-801 binding in a range of concentrations of L-glutamate and a constant concentration of [^{3}H]MK-801 (10 nM). Concentration of L-glutamate was as follows: (A), 0.0 μM; (B), 0.25 μM; (C), 2.5 μM; (D), 10 μM. (A) Note that only background levels of binding were present in sections incubated without L-glutamate. (B-D) As the concentration of L-glutamate increased, [3H]MK-801 binding increased in a dose-dependent manner. Autoradiographs are shown in inverse exposure so that white indicates high density of binding and black indicates low levels of binding.


A
B
C
D

Discussion

The lateral striatum (LSTR), entorhinal cortex (ERC) and inner frontal cortex (IFCTX) were the three brain regions that were shown in Chapter 3 to undergo an age-related decrease in [^{3}H]MK-801 binding when maximal levels of glutamate, glycine and spermine were present. These regions also showed the greatest percentage decrease in E_{max} values without concomitant changes in EC_{50} values as a function of age. Because both middle-aged and aged animals displayed a significant decrease in E_{max} values without differences in their EC_{50} values, it appears that there is an age-related effect on the maximum density of [^{3}H]MK-801 bound as a function of L-glutamate concentration.

The maximal effect that L-glutamate has on stimulating [^{3}H]MK-801 binding is decreased in aged rats. This age-dependent decrease in E_{max} may be the result of a down-regulation of NMDA receptors in specific brain regions. These brain regions may undergo down-regulation to protect against excitotoxic damage. In a developmental study, Oster and Schramm (1993) demonstrated that the process of down-regulation of NMDA receptor activity occurs in rat cerebellar granule cells. In these experiments, NMDA was added to cell cultures derived from postnatal day 8 rats. This resulted in suppression of NMDA receptor-mediated ^{45}Ca uptake without affecting the viability or total cell protein of the cultured neurons. The down-regulation also rendered the neurons

resistant to NMDA toxicity. They proposed that a similar form of down-regulation may play a role in adjusting the activity of postsynaptic NMDA receptors following synaptogenesis.

Jakoi et al. (1992) showed that activation of EAA receptors in cultured hippocampal neurons caused a down-regulation of the protein ligatin at both physiologic and excitotoxic levels of glutamate stimulation. This down-regulation was shown to be mediated by the NMDA receptor and it was hypothesized that EAA receptor activation may alter expression of NMDA receptors. This effect on NMDA receptor expression may be a central mechanism that underlies some of the long-lasting functional and pathophysiological effects of EAA receptor activation on cell function (Jakoi, et al., 1992). Therefore, it is possible that down-regulation of NMDA receptors in the aging CNS may affect the efficacy of L-glutamate to maximally enhance the receptor's binding capacity in the aged brain.

Neurons bearing NMDA receptors are vulnerable to excitotoxic injury associated with an excessive concentration of extracellular glutamate or related agonists (Choi, 1987). An age-related increase in basal glutamate release in mouse striatal and hippocampal slices has been reported (Freeman and Gibson, 1987). Low affinity glutamate uptake into rat cerebral cortical slices (Matsumoto et al., 1982) and brain mitochondria (Victorica et al., 1985) has been shown to be reduced as a function of age. Furthermore, an age-related

loss in the number of high affinity glutamate transport (uptake) sites of rat striatal (Price et al., 1981) and cortical synaptosomes (Wheeler and Ondo, 1986) has been reported. Taken together, these reports suggest that extracellular glutamate levels are elevated with aging. Therefore, the age-related down-regulation in NMDA receptors may essentially be a functional trade-off in order to protect cells bearing NMDA receptors from excitotoxic insult.

A recent study reported data that contradict some of the findings here (Serra et al., 1994). Using hippocampal, striatal and cerebral cortical brain homogenates from 3-, 18- and 24-month-old male Wistar Kyoto rats a decrease (20 to 25%) was seen in the total number of NMDA receptors in 18- and 24-month-old rats. No significant differences were found in K_D between young and aged rats. In addition, no difference was seen in the sensitivity of [^{3}H]MK-801 binding as a function of glutamate concentration in the cerebral cortex, striatum or hippocampus of the aged rats. However, in the hippocampus of 18-month-old rats, glycine and glutamate stimulated [^{3}H]MK-801 with a higher efficacy than in 3- or 24-month-old rats. This group speculated that the loss of NMDA receptors in the hippocampus of 18-month-old rats is counteracted by a physiological compensatory mechanism. This mechanism somehow increases NMDA receptor activity and thereby prevents a decline in cognitive function in the "early phase of aging" (e.g., 18-months-of-age). The compensation ceases to function adequately during, what this

group termed, the "late phase of aging" (i.e. 24 months of age) (Serra et al., 1994). One possible explanation for the differences in these findings is that 18-month-old animals were not examined in the present study. Although Serra et al. (1994) used thorough washing techniques on the rat membrane homogenates they speculated that the sensitivity of [^{3}H]MK-801 binding to endogenous concentrations of agonists and allosteric modulators of the NMDA receptor may have affected their results. Therefore, another possible explanation for this discrepancy is the aging-induced changes in the brain concentrations of glycine and glutamate and/or other modulators which could affect [^{3}H]MK-801 binding (Freeman and Gibson, 1987).

Gonzales et al. (1991) found an age-related decrease in NMDA receptor function in aged animals by analyzing NMDA-stimulated neurotransmitter release in rat cortex, hippocampus and striatum. They showed that NMDA-stimulated [^{3}H]NE (norepinephrine) and [^{3}H]DA (dopamine) release were decreased as a function of age. In a similar study, Pittaluga et al. (1993) showed an age-related decrease in NMDA receptor-mediated noradrenaline release in rat hippocampus.

It should be noted that there are advantages to using quantitative autoradiographic analysis over brain tissue homogenates in that it allows for a more detailed analysis of any anatomical distributional changes occurring in the rat CNS. However, although there are other methods that can also

test the NMDA receptor's functional capacity (e.g. electrophysiological patch clamping techniques, molecular biological oocyte expression systems etc.), the experiments performed in this study tested the ability of glutamate to enhance [^{3}H]MK-801 binding. It should be noted that a confounding variable possibly influencing these results is the phenomenon of receptor desensitization, which is defined as the lack or decline of a response as a result of previous activation. Since brain slices in these experiments were exposed to glutamate for long periods of time, the receptors may have desensitized. Therefore, responses of NMDA receptors may have been examined in the desensitized state.

The sections used in this study were rinsed for a total of 60 minutes at 30^{o}C prior to incubation with [^{3}H]MK-801. Previous studies performing [^{3}H]MK-801 binding assays have shown that prewashing sections for as little as thirty minutes removed substantial amounts of endogenous amino acids since [^{3}H]MK-801 binding following incubation was stimulated significantly with the addition of exogenous glutamate (Sakurai et al., 1990). Therefore, any potential effects of desensitization were probably not seen prior to the incubation with radiolabeled MK-801. It should also be noted that the time-course for the amount of [^{3}H]MK-801 bound to reach equilibrium in the binding assay occured prior to the one hour incubation period utilized in these experiments (the incubation step is when the brain sections are exposed to [^{3}H]MK-801 in the presence of glutamate, glycine and

spermine). Since, at equilibrium, the receptors have reached their greatest maximal response, comparisons can be made between the various age groups with any observed differences in the density of [^{3}H]MK-801 binding being attributed to the age factor. Monaghan (1991) examined the differential stimulation of [^{3}H]MK-801 binding to subpopulations of NMDA receptors and stated that since excess concentrations of glycine and spermine were present in all incubations and both sites analyzed could be 'activated' neither site appeared to be in a desensitized form. However, if desensitization occurs within minutes or seconds of glutamate application, then age-related differences in [^{3}H]MK-801 binding may not be able to be interpreted as clearly due to potential age-related differences in densensitization mechanisms. In order to alleviate some of these potential problems in these experiments, desensitization will be defined as a decreased density of [^{3}H]MK-801 (response) as a function of increased glutamate concentration. It is important to note that the density of [^{3}H]MK-801 bound did not decrease after reaching maximal levels in any of the age groups, suggesting that desensitization may not be a significant factor in these studies.

Consistent with L-glutamate's ability to open the ion channel associated with the NMDA receptor, it was shown in this study that [^{3}H]MK-801 binding increased as a function of L-glutamate concentration in all age groups. However, these findings suggest that there is an effect of age on the

maximal increase in [^{3}H]MK-801 binding induced by L-glutamate. These age-dependent changes in the maximal response elicited by L-glutamate may be due to some functional alteration in the NMDA receptor/channel complex. Hollenberg (1985) proposed that receptors can be modified by reactions leading to either covalent or noncovalent bond formation. In covalent modifications, reactions involving receptor phosphorylation, disulfide-sulfhydryl exchange and receptor proteolysis are all possible mechanisms involved in receptor function (Hollenberg, 1985). Non-covalent interactions would include changes in membrane potential, receptor distribution (patching, capping), allosteric interactions involving either protein-protein (e.g. mobile receptor model) or small ligand (cations, anions, nucleotides, etc.) receptor interactions, and alterations in the membrane lipid environment (e.g. lipid methylation or hydrolysis) (Hollenberg, 1985). All of these processes could result in changes in the functional modification of the NMDA receptor. In conclusion, there is probably a combination of both an age-dependent decrease in the total number of NMDA receptors available for binding as well as some age-related change in the functional capacity of the remaining NMDA receptors. This may account for some of the differences found in the maximal increase in [^{3}H]MK-801 induced by L-glutamate as a function of age.

CHAPTER 5
AGE-RELATED CHANGES IN THE LEVELS OF mRNA CODING FOR SPECIFIC NMDA RECEPTOR SUBUNITS IN THE CNS OF F-344 RATS.

Introduction

Two NMDA receptor subunit families have been cloned and are named NMDAR1 (NR1) and NMDAR2 (NR2)(Monyer et al., 1992; Moriyoshi et al., 1991). NMDAR1 has at least eight alternatively spliced forms and these are differentiated from each other by an insertion at the extracellular amino-terminal region, deletion at two carboxy-terminal regions, or by combinations of both (Moriyoshi et al., 1991). NMDAR1 and its isoforms have been shown in *Xenopus* oocyte expression systems to exhibit electrophysiological and pharmacological responses characteristic of the NMDA receptor. These include agonist and antagonist selectivity, glycine modulation, permeability to calcium, voltage-dependent channel block by magnesium as well as inhibition by zinc (Moriyoshi et al., 1991).

NMDAR2 subunit family members have been shown to potentiate the electrophysiological responses of NMDAR1 but are not functional in homomeric configurations with each other. NMDA-induced currents in oocytes expressing NR1 and NR2A, NR2B or NR2C are approximately 100 times larger than they are in oocytes expressing homomeric NR1 channels. It

should be noted that these currents more closely resemble native NMDA receptors and thus native NMDA receptors probably represent heteromeric configurations formed from NR1 subunits and members of the NR2 subunit family (Monyer et al., 1992).

The NMDA receptor has been implicated in age-related learning and memory deficits in humans as well as other animals (Barnes, 1979; Barnes and McNaughton, 1985). Detailed *in situ* hybridization analyses can now be performed due to the recent isolation of functional cDNA clones for the rat NMDA receptor NR1 (NMDAR1), splice variants of NR1 as well as for the members of the NR2 subunit family (i.e., NR2A, NR2B, NR2C and NR2D) (Buller et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monaghan et al., 1993; Monyer et al., 1992; Moriyoshi et al., 1991; Nakanishi, 1992). In this study, this technique was utilized to examine mRNA coding for the NMDA receptor subunits in the aged brain.

Methods

In situ Hybridization

Oligonucleotide probes for this study (45 mer) were constructed from published sequences and specific to NMDAR1, four alternatively spliced versions of NMDAR1 (NR1-not insert 1 or $NR1_{0xx}$; NR1-insert 1 or $NR1_{1xx}$; NR1-insert 2 or $NR1_{x1x}$; and NR1-insert 3 or $NR1_{xx1}$), and the members of the NMDAR2 subunit family (NR2A, NR2B, NR2C, and NR2D). The probes for this study were graciously supplied by Dr. Daniel T.

Monaghan, University of Nebraska Medical Center, Omaha NE. See Chapter 2 for details on the probe labeling procedure, tissue sectioning, fixation and hybridization.

Data Analysis

Computer assisted semi-quantitative densitometric measurements were performed as described in detail in Chapter 2. Six-month (young), twelve-month (middle-aged) and twenty-four month (aged) F-344 rat brains were used (n=6 for each age group). The outer frontal cortex (OFCTX), inner frontal cortex (IFCTX), lateral septum (LSEP), lateral striatum (LSTR), lateral thalamus (LTHAL), entorhinal cortex (ERC), dentate gyrus (DG), and hippocampal areas CA1 and CA3 were the brain regions analyzed in this study. Relative mRNA density levels were obtained for each of the oligonucleotide NMDA probes. Density values from young adults were compared to the middle-aged and aged animals.

Statistical Analysis

Six blocks of animals, each block consisting of an animal from each age group, were assessed. The experimental design consisted of a within-subject factor with nine levels (brain region), a quantitative between-subject fixed factor with three levels (age), and each of six levels (experimental day). Repeated measures analysis of variance (ANOVA) with between-subject randomized blocks and age considered as a linear covariate were used to model the density of mRNA per

oligonucleotide probe as a function of age within each brain region and to determine if this pattern differed significantly among brain regions.

Results

Significant age-related changes were found in NMDAR1, as well as three of the splice variants of NMDAR1: $NR1_{0xx}$, $NR1_{1xx}$, and $NR1_{x1x}$ (Figures 5-1,5-2,5-4,5-5 and 5-7). More specifically, NMDAR1 mRNA measured in 12-month-old rats showed a significant decrease in the outer frontal cortex (17.8%), inner frontal cortex (15.0%), hippocampal area CA3 (9.0%) and the lateral striatum (14.1%). In 24-month-old rat brains, there was a significant decrease seen in all brain regions analyzed, with the exception of the molecular layer of the dentate gyrus. Areas showing the greatest percent decline included the entorhinal cortex (28.1%) > lateral thalamus (24.2%) > lateral striatum (20.7%) > outer frontal cortex (18.6%) > inner frontal cortex (17.7%) > hippocampal areas CA3 (14.5%) and CA1 (14.4%) > lateral septum (10.4%).

Relative mRNA density for NMDAR1 splice variant $NR1_{0xx}$ (not containing the 21 amino acid N-terminal insert) showed a statistically significant decrease in the entorhinal cortex (5.6%) as well as the CA3 region of the hippocampus (9.2%) in 24-month-old animals. This splice variant also showed a significant increase in mRNA density in hippocampal area CA1 (5.5%) from 12-month-old animals. There was a significant age-related change in relative mRNA density for two other

splice variants of NMDAR1. The $NR1_{1xx}$ (those NMDAR1 isoforms containing the N-terminal insert) showed an increase in the lateral septum (9.6%) from 12-month-old animals when compared to 6-month-old animals (Figure 5-5). $NR1_{x1x}$ isoforms (those splice variants containing the first of the two C-terminal inserts) also showed an increase in the lateral septum (5.3%) along with the lateral striatum (3.4%) and lateral thalamus (4.7%) from 12-month-old animals (Figure 5-7). In contrast, no significant differences were observed in either $NR1_{1xx}$ or $NR1_{x1x}$ mRNA density in any of the brain regions analyzed from 24-month-old animals (Figures 5-5, 5-6, 5-7, and 5-8). The density of NMDAR1 splice variant $NR1_{xx1}$ (containing the second C-terminal insert) did not show any differences in any of the brain regions analyzed at any age (Figures 5-9 and 5-10).

The NMDAR2 subunit family members, NMDAR2A and NMDAR2B, did not show any age-related differences in relative density of mRNA (Figures 5-11, 5-12, 5-13 and 5-14). NMDAR2C subunit mRNA was not present in any of the brain regions examined. Since this subunit is found only in the cerebellum it was excluded from these analyses. There was no detectable NMDAR2D mRNA present in any of the age groups. This may be due to the fact that this subunit appears to be developmentally regulated such that there is an apparent shift in relative expression of NMDAR2D to NMDAR2B expression at about six months-of-age in the rat CNS. Representative autoradiograms are seen in Figures 5-15, 5-16 and 5-17.

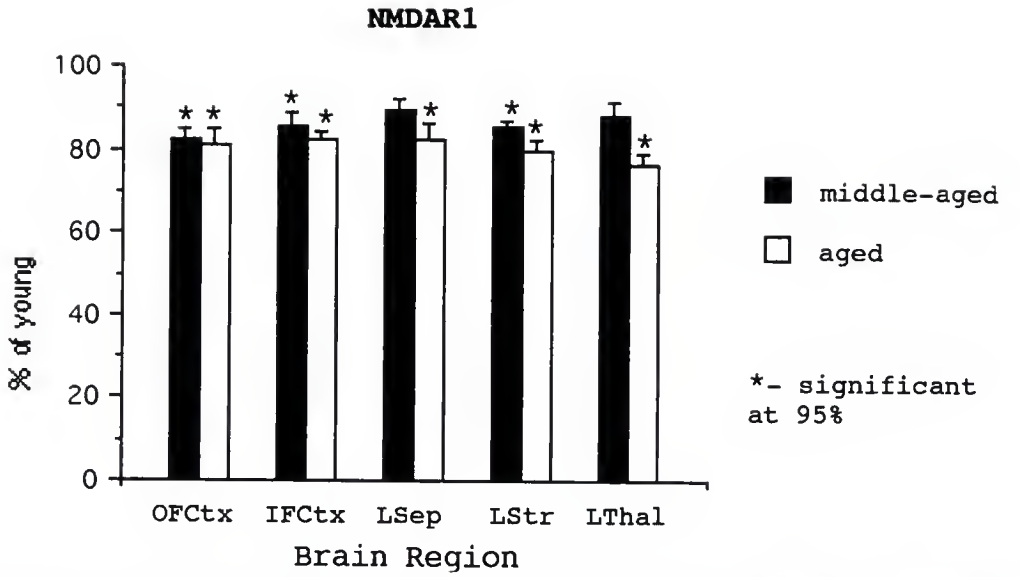


Figure 5-1. *In situ* hybridization analyses of NMDAR1 mRNA in cortical and subcortical brain regions. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals.(OFCtx=outer frontal cortex; IFCtx=inner frontal cortex; LSep= lateral septum; LStr= lateral striatum; LThal= lateral thalamus).

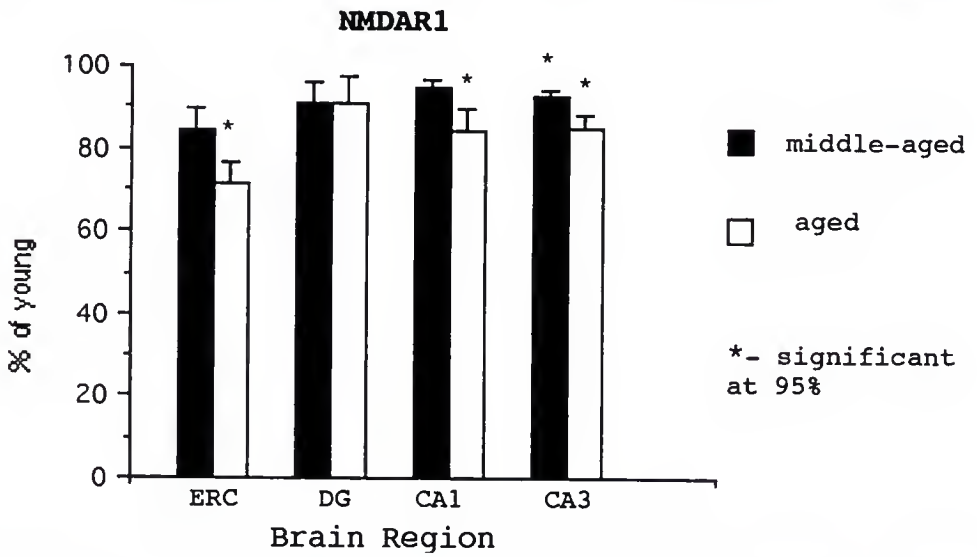


Figure 5-2. *In situ* hybridization analyses of NMDAR1 mRNA in hippocampus and entorhinal cortex. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC= entorhinal cortex; DG= molecular layer of the dentate gyrus; CA1 & CA3= hippocampal areas).

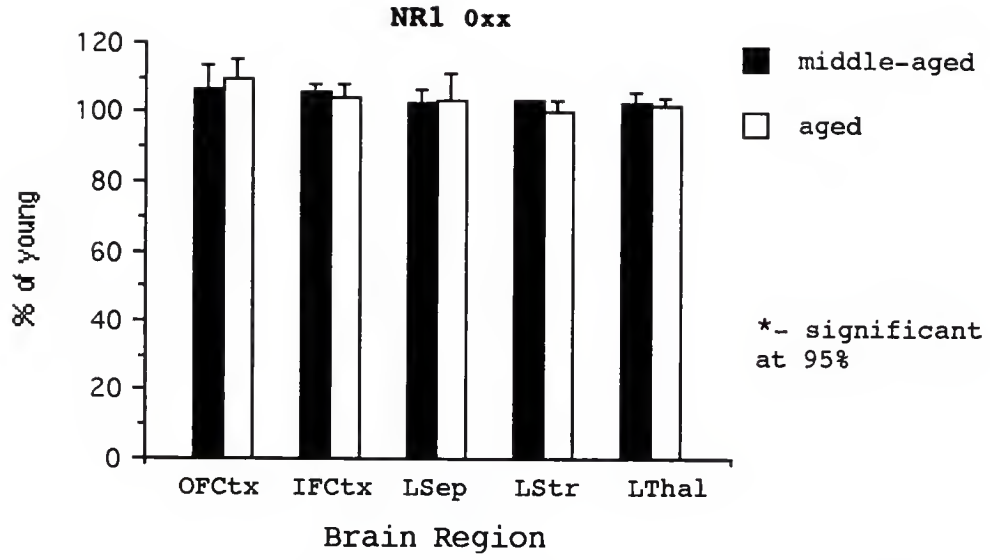


Figure 5-3. *In situ* hybridization analyses of NMDAR1 splice variant $NR1_{0xx}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep=lateral septum;LStr=lateral striatum; LThal=lateral thalamus).

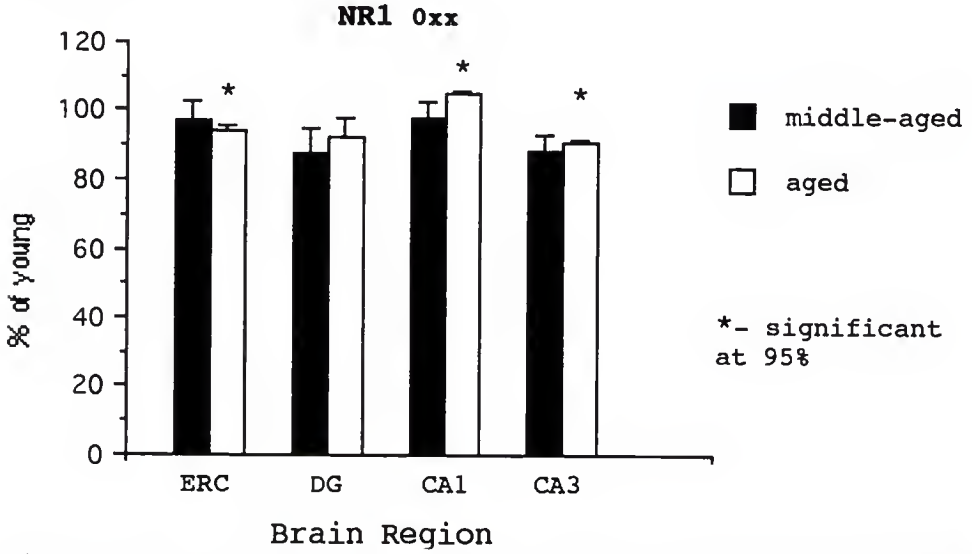


Figure 5-4. *In situ* hybridization analyses of the NMDAR1 splice variant $NR1_{0xx}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC=entorhinal cortex;DG= molecular layer of the dentate gyrus;CA1 & CA3=hippocampal areas).

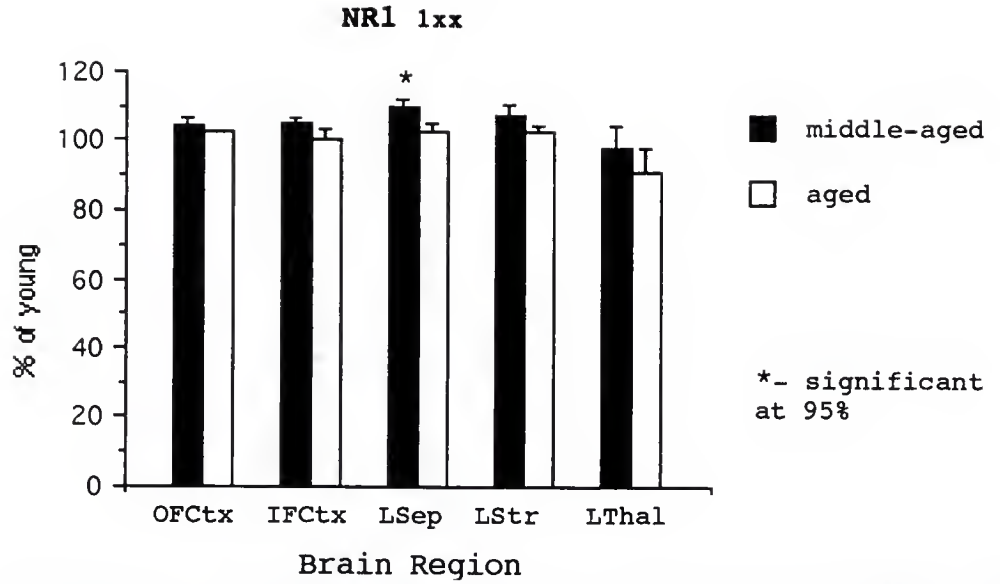


Figure 5-5. *In situ* hybridization analyses of NMDAR1 splice variant $NR1_{1xx}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep= lateral septum;LStr= lateral striatum; LThal=lateral thalamus).

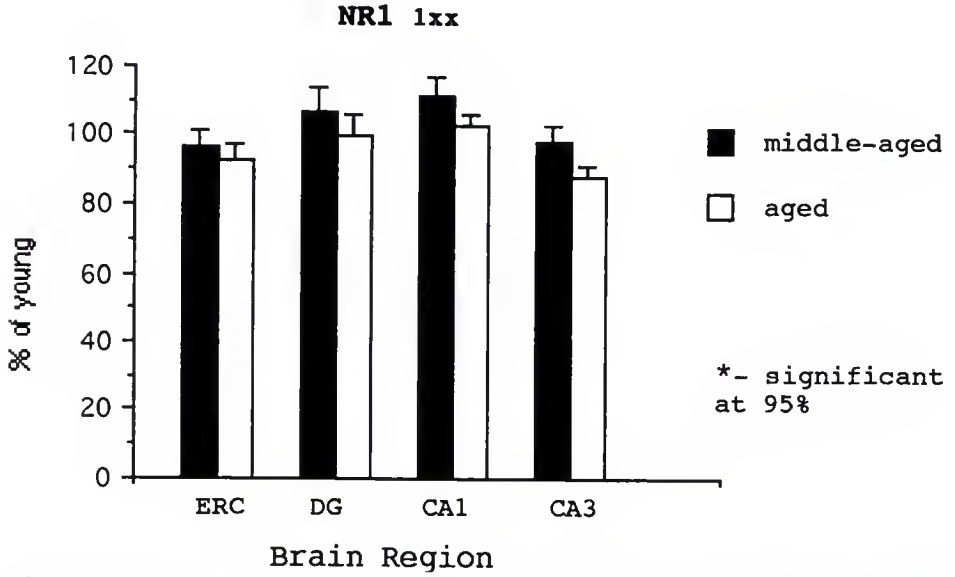


Figure 5-6. *In situ* hybridization analyses of the NMDAR1 splice variant $NR1_{1xx}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC=entorhinal cortex; DG= molecular layer of the dentate gyrus;CA1 & CA3= hippocampal areas).

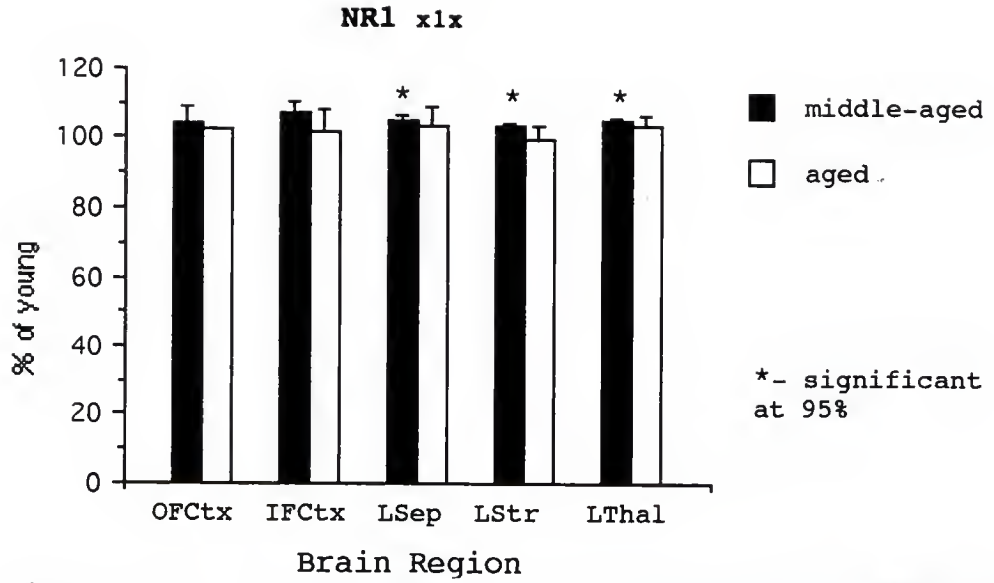


Figure 5-7. *In situ* hybridization analyses of NMDAR1 splice variant $NR1_{x1x}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep=lateral septum;LStr=lateral striatum; LThal=lateral thalamus).

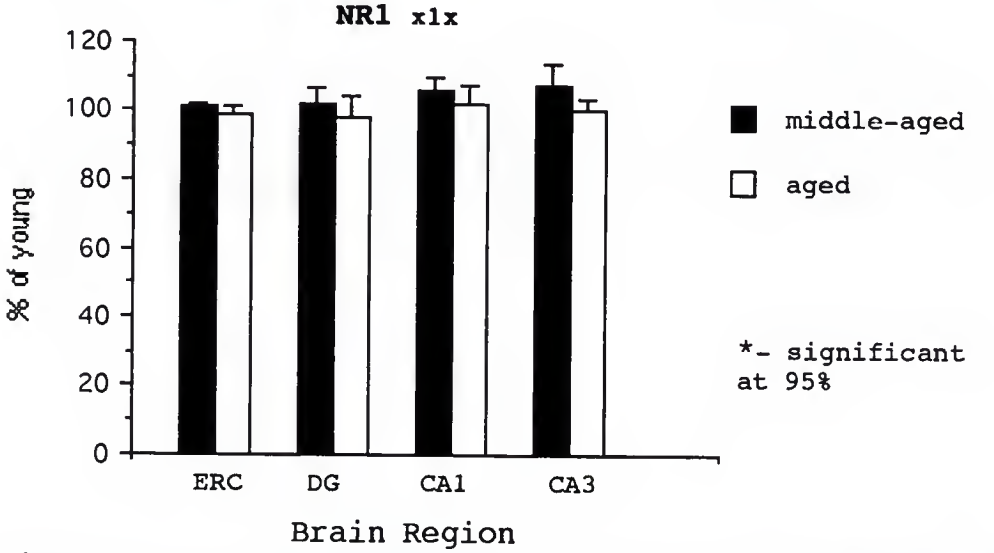


Figure 5-8. *In situ* hybridization analyses of the NMDAR1 splice variant $NR1_{x1x}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC=entorhinal cortex; DG=molecular layer of the dentate gyrus;CA1 & CA3=hippocampal areas).

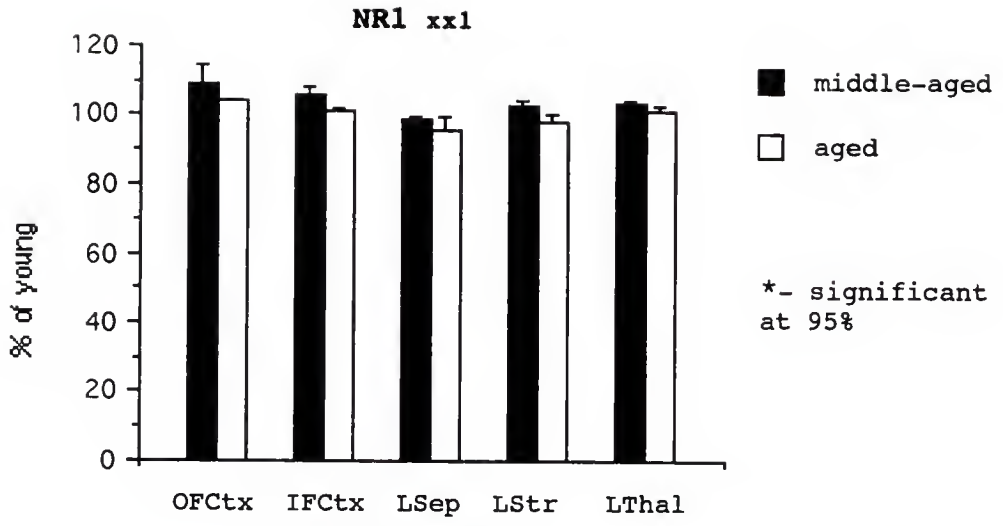


Figure 5-9. *In situ* hybridization analyses of NMDAR1 splice variant $NR1_{xx1}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep=lateral septum;LStr=lateral striatum; LThal=lateral thalamus).

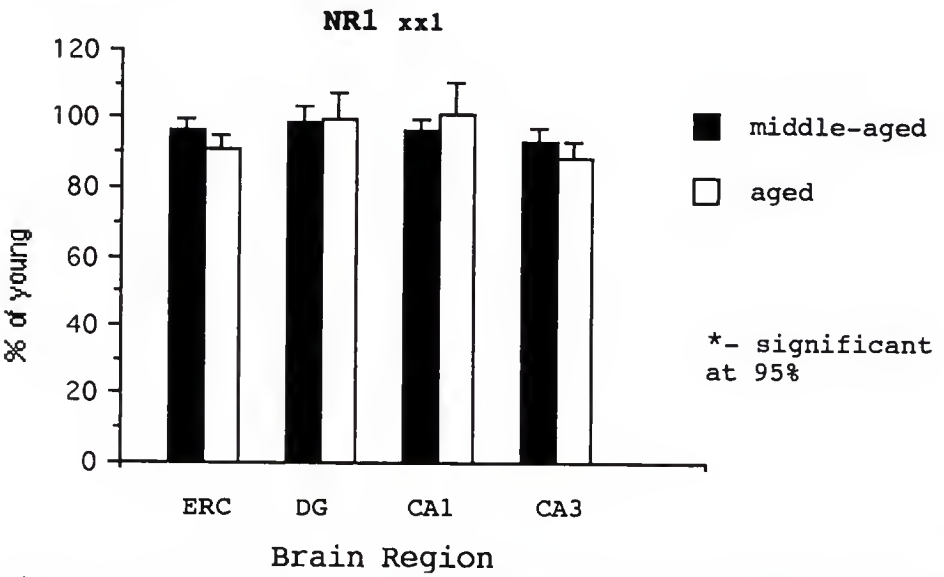


Figure 5-10. *In situ* hybridization analyses of the NMDAR1 splice variant $NR1_{xx1}$ mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC=entorhinal cortex;DG= molecular layer of the dentate gyrus;CA1 & CA3=hippocampal areas).

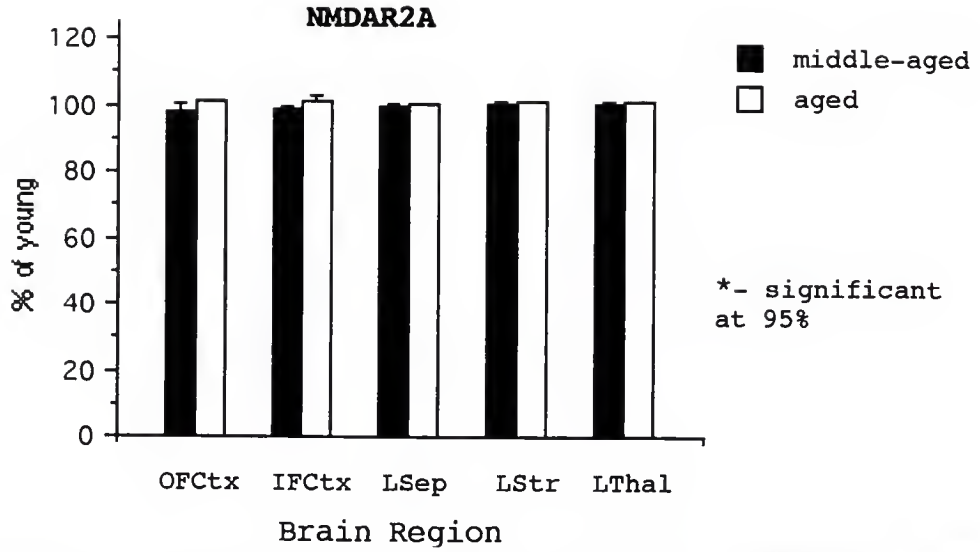


Figure 5-11. *In situ* hybridization analyses of NMDAR2A mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep= lateral septum;LStr= lateral striatum;LThal= lateral thalamus).

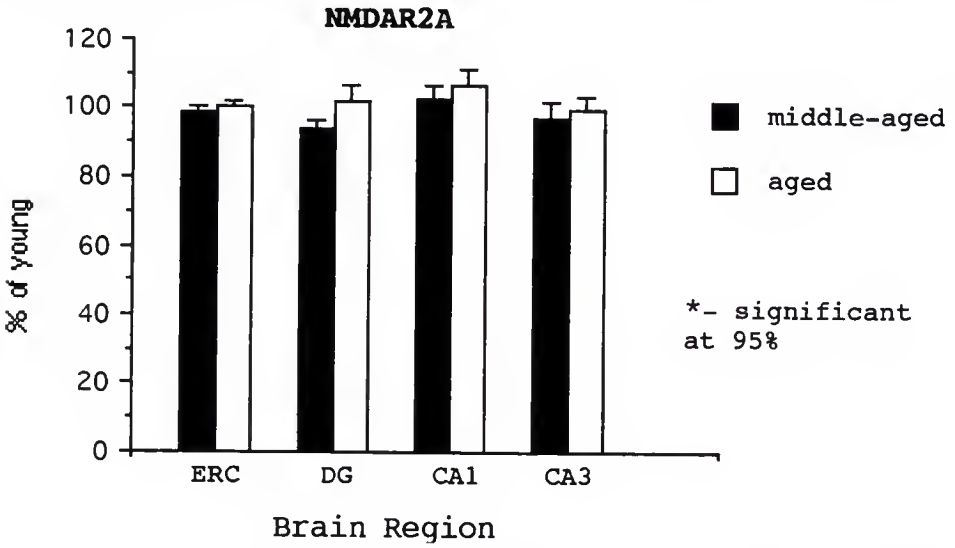


Figure 5-12. *In situ* hybridization analyses of NMDAR2A mRNA. (ERC=entorhinal cortex; DG=molecular layer of the dentate gyrus;CA1 & CA3=hippocampal areas). All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals.

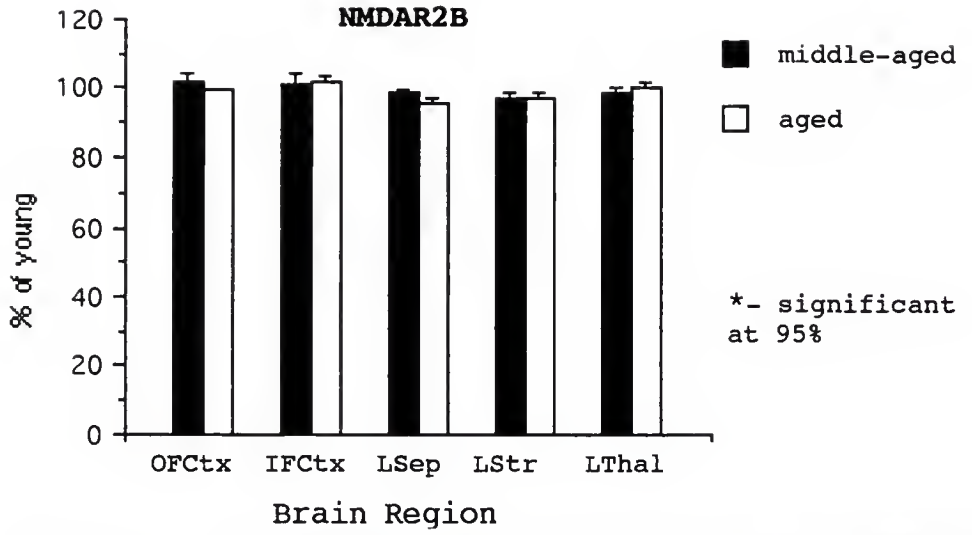


Figure 5-13. *In situ* hybridization analyses of NMDAR2B mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (OFCtx=outer frontal cortex;IFCtx=inner frontal cortex;LSep= lateral septum;LStr=lateral striatum;LThal=lateral thalamus).

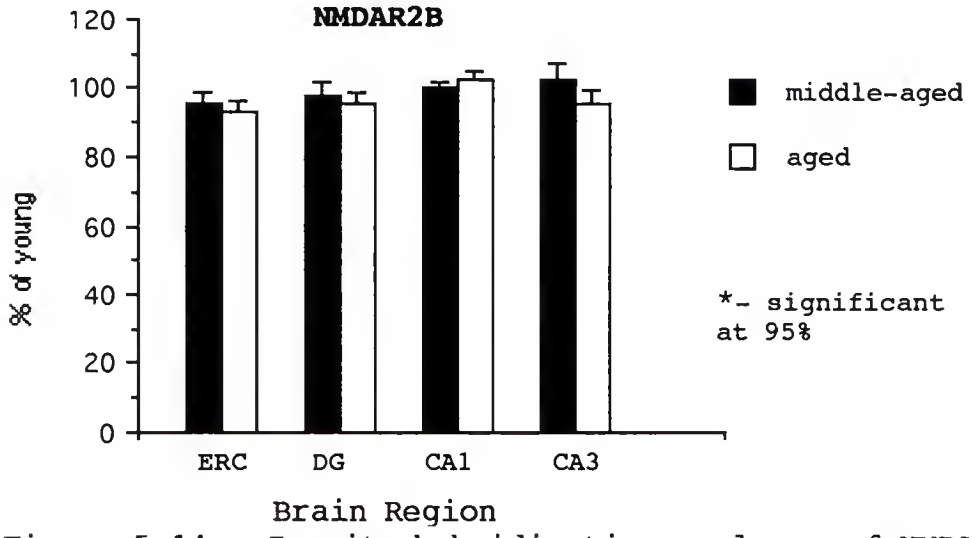


Figure 5-14. *In situ* hybridization analyses of NMDAR2B mRNA. All data are the mean ± S.E.M. Asterisk indicates densitometric values significantly different than young animals. (ERC=entorhinal cortex;DG= molecular layer of the dentate gyrus;CA1 & CA3=hippocampal areas).

Figure 5-15. The distribution of mRNA specific to NMDAR1 in A) 24-month-old; B) 12-month-old; and C) 6-month-old rat brain.

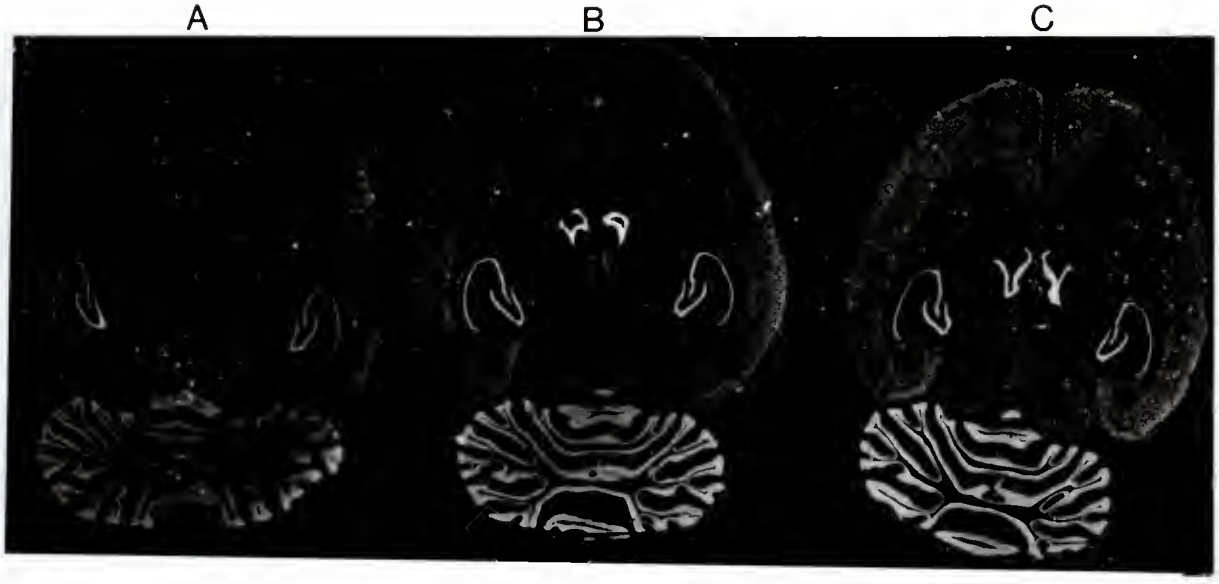

A
B
C

Figure 5-16. The distribution of mRNA specific to: (A) $NMDAR1_{0xx}$ (not insert1); (B) $NMDAR1_{1xx}$ (insert1) in 6-,12- and 24-month-old rat brain.

A

B

Figure 5-17. The distribution of mRNA specific to (A) $NMDAR1_{x1x}$ (insert2) and (B) $NMDAR1_{xx1}$(insert3) in 6-,12- and 24-month-old rat brain.

A

B

Figure 5-18. The distribution of mRNA specific to A) NMDAR2A and (B) NMDAR2B in 6-,12- and 24-month-old rat brain.

A

B

Discussion

In summary, results from this study demonstrate that in virtually all brain regions examined, mRNA for the NMDAR1 subunit was decreased in aged animals. NMDAR1 mRNA was also shown to decrease in the outer and inner frontal cortex, CA3 region of the hippocampus and lateral striatum of middle-aged animals. These data are consistent with many studies that have shown a widespread decrease in the density of the NMDA receptor channel complex in aged animals as well as humans (Anderson et al., 1989; Ingram et al., 1992; Magnusson and Cotman, 1993; Peterson and Cotman, 1989; Piggott et al., 1992; Serra et al., 1994; Tamaru et al., 1991; Wenk et al., 1991). A decrease in NMDA receptor density may be due to a decrease in the mRNA coding for the main subunit of the NMDA receptor.

The splice variants that do not contain the N-terminal insert ($NR1_{0xx}$) were shown to decrease in the entorhinal cortex (ERC) and the CA3 region of the hippocampus of aged animals. It is possible that the decrease in [^{3}H]MK-801 binding (Chapter 3) in the entorhinal cortex may be due to a loss in the NMDAR1 subunit and in the splice variant $NR1_{0xx}$.

Interestingly, an increase in the density $NR1_{0xx}$ mRNA in the CA1 region of the hippocampus in aged animals was found, with no increase in the NMDAR1 mRNA. Rather, there was a significant decrease in NMDAR1 mRNA in this same brain

region. One explanation for this observation may be that this particular brain region is upregulating the specific NMDAR1 isoform, $NR1_{0xx}$, to compensate for decreased levels of NMDAR1 subunit in an attempt to maintain NMDA receptor/channel neurotransmission in aged CNS. In support of this, splice variants lacking the N-terminal insert, i.e. $NR1_{0xx}$, exhibit a 3- to 5-fold higher affinity for agonist when expressed in *Xenopus* oocytes (Durand et al., 1993). It has been suggested that since the glutamate binding site is presumably contained within the extracellular N-terminal domain of the NMDA receptor (although not at the 21-amino acid insert) the effect on agonist affinity may be the result of a delocalized conformational change in the N-terminal domain caused by the insert (Durand et al., 1993). Therefore, an upregulation of this specific NMDAR1 isoform may allow the aging CA1 region of the hippocampus to better maintain an optimal degree of neurotransmission. Alternatively, a decrease in this isoform in brain regions such as the ERC and CA3, may lead to an enhanced deficit in NMDA receptor-mediated neurotransmission.

A similar argument can be proposed to explain variants of NMDAR1 that underwent an increase in mRNA density. The NMDAR1 splice variant with the N-terminal insert ($NR1_{1xx}$) and the splice variant containing the first of the two C-terminal inserts ($NR1_{x1x}$) showed a significant increase in the lateral septum of 12-month-old rats. There were no significant changes in the same brain region of 24-month-old rats . The

splice variant $NR1_{x1x}$ also showed an increase in the lateral striatum (LSTR) and the lateral thalamus (LTHAL) in middle-aged animals. It is possible that middle-aged animals are better able to upregulate these isoforms in these brain regions where the aged animals cannot, due to an age-related change in some aspect of transcriptional control. However, Worley et al. (1993) showed that identical transcription factor responses to various stimulus patterns were observed in 6- to 12-month-old and 23- to 26-month-old rats. They suggested that the synaptic mechanisms involved in the induction of various transcription factors was preserved in aged animals. It should be noted that the stimulus parameters that are used to induce transcription factors are more intense than are required to produce long-lasting synaptic enhancement (LTP) (Barnes, 1979). This suggests a possible dissociation between the conditions sufficient for LTP induction and those necessary for induction of transcription factors (i.e., c-*fos*, c-*jun*, *zif268*). Previous studies have demonstrated that the induction of LTP is comparable in young and aged rats, whereas the rate of decay of the enhanced synaptic response is accelerated in aged animals (Barnes, 1979; deToledo-Morrell and Morrell, 1985). Therefore, this acceleration of decay of LTP may account for some of the age-dependent differences in this form of synaptic plasticity.

The NMDAR1 receptor splice variants that contain the N-terminal insert show a lower agonist affinity and little or

no spermine potentiation at saturating glycine concentrations (Durand et al., 1993). A possible explanation for an increase in $NR1_{1xx}$ could be that the 12-month-old animals are upregulating this isoform in specific brain regions to enhance the NMDA receptor channel's affinity for agonist. It has been shown that the NMDA receptor channel complex can be formed by heteromeric as well as homomeric configurations between the NMDAR1 subunit as well as its splice variants (Monyer et al., 1992).

The stability of the mRNA levels for the NMDAR2 family in aged animals may also represent a compensatory mechanism to maintain the functionality of the NMDA receptor complex. NMDAR2 family members are known to potentiate NMDA receptor neurotransmission when expressed in heteromeric combinations NMDAR1 (Monyer et al., 1992). It should be noted that NMDAR2 subunits do not form functional receptors when expressed in homomeric combinations (Monyer et al., 1992). This may indicate that the NMDAR1 subunit and its various isoforms confer a more important functional role in NMDA receptor-mediated neurotransmission when compared to NMDAR2 subunits. This may explain why only changes in mRNA coding for NMDAR1 and/or its splice variants were detected as a function of age.

It should be noted that the presence of NMDAR1 or NMDAR2 subunit mRNA in particular CNS neurons may not necessarily indicate NMDA receptor protein is translated. Sucher et al. (1994) showed the presence of endogeneous NMDAR1 transcripts

without receptor protein in PC12 cells suggesting that the expression of the NMDAR1 protein may be controlled by post-transcriptional mechanisms. A study of the density of NMDAR1 and NMDAR2 protein utilizing antibodies raised against the NMDA receptor subunits is performed in Chapter 6.

In conclusion, the results of these experiments have demonstrated changes in mRNA coding for the NMDA receptor channel complex in the aged brain. These results, in combination with immunocytochemical and ligand binding studies, have contributed to further characterization of the NMDA receptor in the aged brain.

CHAPTER 6
AGE-RELATED EFFECTS ON NMDAR1 AND NMDAR2A/B PROTEIN LEVELS IN F-344 RAT BRAIN UTILIZING IMMUNOHISTOCHEMISTRY

Introduction

N-methyl-D-aspartate (NMDA) receptor-mediated glutamatergic neurotransmission has been shown to be important in the formation of learning and memory in animals and humans (Danysz et al., 1988; DeToledo-Morrell et al., 1984; Morris et al., 1986). NMDA receptor density has been shown to undergo a significant region-specific decrease in aged animals (Anderson et al., 1989; Ingram et al., 1992; Magnusson and Cotman, 1993; Peterson and Cotman, 1989; Piggott et al., 1992; Serra et al., 1994; Tamaru et al.,1991; Wenk et al., 1991; see Chapter 3). These previous studies examined the NMDA receptor utilizing various ligand binding techniques. Recently, two NMDA receptor subunit families were cloned and named NMDAR1 (Moriyoshi et al., 1991) and NMDAR2 (Monyer et al., 1992). NMDAR1 has at least eight alternatively spliced forms. NMDAR1 as well as all of its isoforms have been shown in *Xenopus* oocyte expression systems to exhibit electrophysiological and pharmacological responses characteristic of the native NMDA receptor (Moriyoshi et al., 1991). The four members of the NMDAR2 subunit family are named NMDAR2A, NMDAR2B, NMDAR2C and NMDAR2D (Monyer et al.,

1992). In heteromeric combinations with NMDAR1 or splice variants, NMDAR2 subunits have been shown to potentiate electrophysiological responses to agonist but are not functional in homomeric configurations with each other. NMDA-induced currents in *Xenopus* oocytes expressing NMDAR1 and NMDAR2A, NMDAR2B or NMDAR2C are about 100 times larger than homomeric NMDAR1 channels. NMDAR1/NMDAR2 heteromers also exhibit the following properties which are characteristic of native NMDA receptors: 1) voltage-dependent blockade by extracellular Mg^{+2}; 2) high Ca^{+2}/Na^{+} permeability ratio and 3) gating kinetics that are characterized by slow onset and offset time courses to pulses of high concentrations of agonist (Monyer et al., 1992). These data suggest that native NMDA receptors consist of heteromeric configurations formed from NMDAR1 subunits and members of the NMDAR2 subunit family.

Recently, polyclonal antibodies have been raised against the carboxyterminal peptides of both NMDAR1 and NMDAR2. One of the first studies to examine NMDAR1 protein in the rat CNS was performed by Hennegriff et al. (1992). They raised antibodies against the carboxyterminal region of the NMDAR1 receptor cloned by Nakanishi (1991) and examined the regional distribution of NMDAR1 in Western blots from seven brain regions. NMDAR1 was most abundant in neocortex, followed by hippocampus > striatum >> thalamus > olfactory bulb > cerebellum >> brain stem. Petralia et al. (1994a) made a polyclonal antiserum that recognized four of the seven splice

variants of NMDAR1 and showed a wide distribution in the rat CNS. Densely stained cells included the pyramidal and hilar neurons of the CA3 region of the hippocampus, Purkinje cells of the cerebellum and paraventricular neurons of the hypothalamus. Ultrastructural analyses of NMDAR1 antigen showed labeling present in neuronal cell bodies, dendrites and in the postsynaptic densities of synapses. This pattern is consistent with the synthesis, processing and transport of this protein (Petralia et al., 1994a). Staining was not seen in the synaptic cleft. This observation supports an intracellular location of the C-terminus for NMDAR1. The pattern of staining at the tissue level matched that of previous *in situ* hybridization studies but differed somewhat from ligand binding studies (Petralia et al., 1994a).

This same group examined the histological and ultrastructural localization of NMDAR2A and NMDAR2B (Petralia et al., 1994b). They utilized a polyclonal antibody to a C-terminus peptide of NMDAR2A that recognized NMDAR2A and NMDAR2B, and to a lesser extent, NMDAR2C and NMDAR2D. NMDAR2A/B immunostaining was widespread throughout the rat CNS, and the overall distribution resembled NMDAR1 (Petralia et al., 1994a). Many regions contained substantial staining with NMDAR2A/B antibody in neuropil and less staining in neuronal cell bodies. In contrast, NMDAR1 immunostaining was shown to be substantial in neuronal soma and only light to moderate in neuropil (Petralia et al., 1994a). Dense NMDAR2A/B staining was present in postsynaptic densities in

the cerebral cortex and hippocampus, similar to NMDAR1. Since functional NMDA receptors appear to require both NMDAR1 and NMDAR2 subunit proteins for full function, this study provided structural evidence for the coexistence of NMDAR1 and NMDAR2 in the nervous system (Petralia et al., 1994b).

The following experiments utilized the same antisera directed against NMDAR1 and NMDAR2A/B, to examine potential changes in protein levels as a function of increasing age.

Methods

Tissue preparation

Rat brains from 6-,12- and 24-month-old F-344 rats were sectioned on a cryostat in the horizontal plane at 30 μm. One representative brain section from each age group was mounted per slide with group n=5. See Chapter 2 for a more detailed description of the tissue preparation.

Antibody Specificity

See Chapter 2 for a detailed description of antibody specificity. AB1516 (antisera raised against a synthetic peptide corresponding to the C-terminus of rat NMDA receptor subunit) is selective for splice variants NR1-1a,1b,2a,2b (Hollmann et al., 1993). These appear to be the major splice variants expressed in rat brain (Hollmann et al., 1993). It was shown that there was no cross-reaction with other glutamate receptor subunits and the antisera labels a single band corresponding to NMDAR1 in Western blots (Petralia et

al., 1994a). AB1548 (antisera raised against a synthetic peptide corresponding to the C-terminus of rat NMDAR2A receptor subunit) recognizes both NMDAR2A and NMDAR2B subunits equally (Petralia et al., 1994b). This antisera labels a single band corresponding to NMDAR2A and NMDAR2B. No cross reactivity with NMDAR1 or other glutamate receptor subunits was noted (Petralia et al., 1994b).

Immunocytochemical Procedure

See Chapter 2 for a detailed description of this procedure.

Data Analysis

Brain regions analyzed included the outer frontal cortex (OFCTX), inner frontal cortex (IFCTX), lateral septum (LSEP), lateral striatum (LSTR), lateral thalamus, entorhinal cortex (ERC), dentate gyrus (DG) and hippocampal areas CA1 and CA3. Brain regions were compared across varying ages and the relative density of immunostaining was determined by computer assisted densitometry with a Microcomputer Imaging Device, Imaging Research, Inc.(MCID) image processing system. A semi-quantitative measurement of NMDAR1 and NMDAR2A/B immunostaining levels, represented as raw optical densities (ROD), from twelve- and twenty-four-month-old animals in each brain region were normalized with the values obtained for young animals. To reduce variability, all three ages were mounted on one slide with the investigator blind to age.

Statistical Analysis

Five blocks of animals, each block consisting of an animal from each age group, were assessed. A different block of 3 animals, 1 per age group, was assessed on each of 5 experimental days. The experimental design consisted of a within-subject factor with 9 levels (brain region), a quantitative between-subject fixed factor with 3 levels (age), and a between subject random blocking factor with 5 levels (experimental day). Repeated measures analysis of variance (ANOVA) with between-subject randomized blocks and age considered as a linear covariate were used to model the NMDAR1 and NMDAR2A/B immunostaining density data as a linear function of age within each brain region and to determine if this pattern differed significantly among brain regions. The change in NMDAR1 and NMDAR2A/B immunostaining densities per month of age (i.e. the linear slope) was estimated and assessed for statistical significance within each brain region while blocking on experimental day. A general comparison of these slopes among brain regions was then performed by testing for the presence of a significant interaction between age and brain region NMDAR1 and NMDAR2A/B immunostaining effects. Mauchly's sphericity test was used to determine if a multivariate F test (Wilks' Lambda) or a univariate F test should be used for the age x brain region interaction test. In assessing the validity of ANOVA assumptions, it was noted that experimental day age profiles

were relatively parallel within each brain region and that between-subject variability as estimated by the standard deviation was similar among age groups within each brain region. Plots of residuals versus predicted values, residual histograms, and residual normal probability plots were examined to assess goodness of fit in the ANOVA models.

Results

The pattern of immunostaining obtained for both antisera was consistent with previous publications (Petralia et al., 1994a,b). Immunostaining for NMDAR1 was widespread with moderate to high levels present in the OFCTX and IFCTX, ERC, hippocampus, LSTR, LSEP and LTHAL. This widespread expression of NMDAR1 protein matches closely with the distribution of NMDAR1 mRNA (see Chapter 5). There was also widespread expression of NMDAR2A/B protein that mirrored the distribution of NMDAR1 protein (Figure 6-5). This finding is consistent with the fact that native NMDA receptors appear to require both NMDAR1 and NMDAR2A/B subunit proteins for full function (Monyer et al., 1992; Nakanishi 1992). There were no significant age-related differences in NMDAR1 or NMDAR2A/B immunostaining levels in any of the brain regions analyzed (Figures 6-1 through 6-4).

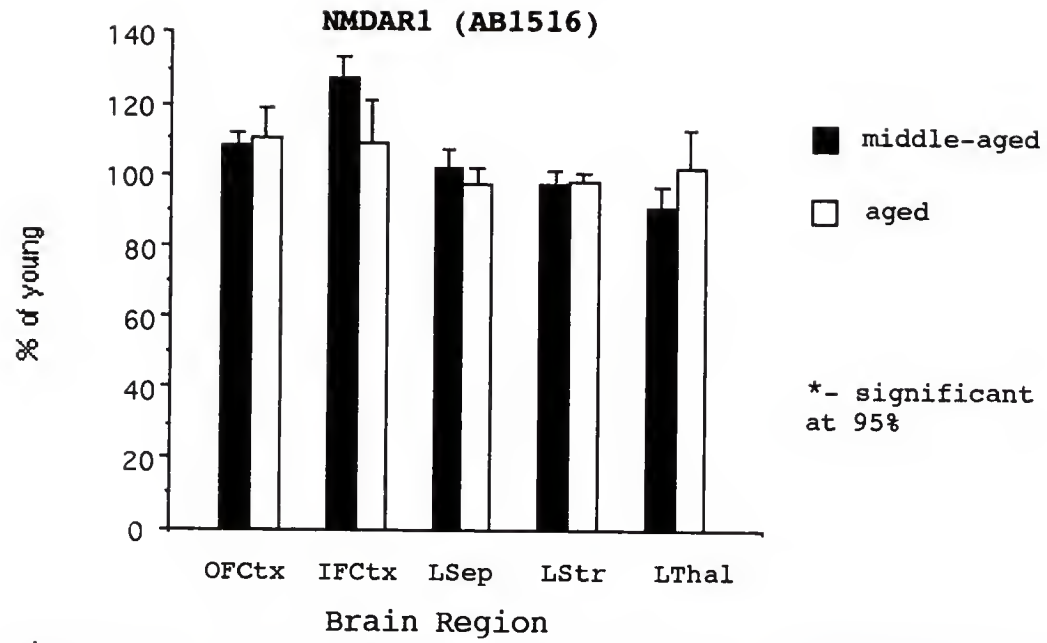


Figure 6-1. Cortical and subcortical brain regions that did not undergo a significant age-related decrease in NMDAR1 antibody staining density. (OFCtx= outer frontal cortex; IFCtx= inner frontal cortex; LSep= lateral septum; LStr= lateral striatum; LThal= lateral thalamus). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate. Data are presented as a percentage of young (6-month-old) animals.

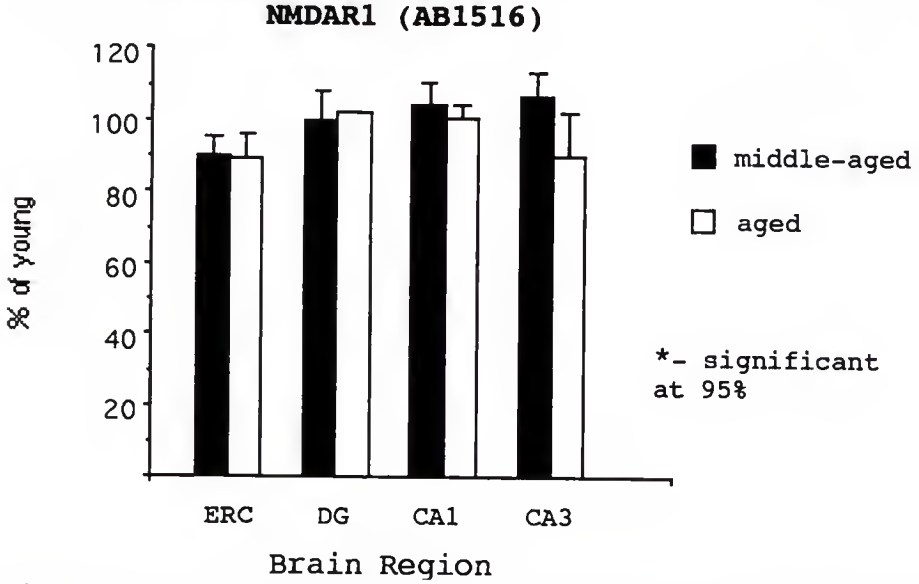


Figure 6-2. Hippocampal and entorhinal cortical brain regions that did not undergo a significant age-related decrease in NMDAR1 antibody staining density. (ERC= entorhinal cortex; DG= molecular layer of the dentate gyrus; CA1= hippocampal area CA1; CA3= hippocampal area CA3). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate. Data are presented as a percentage of young (6-month-old) animals.

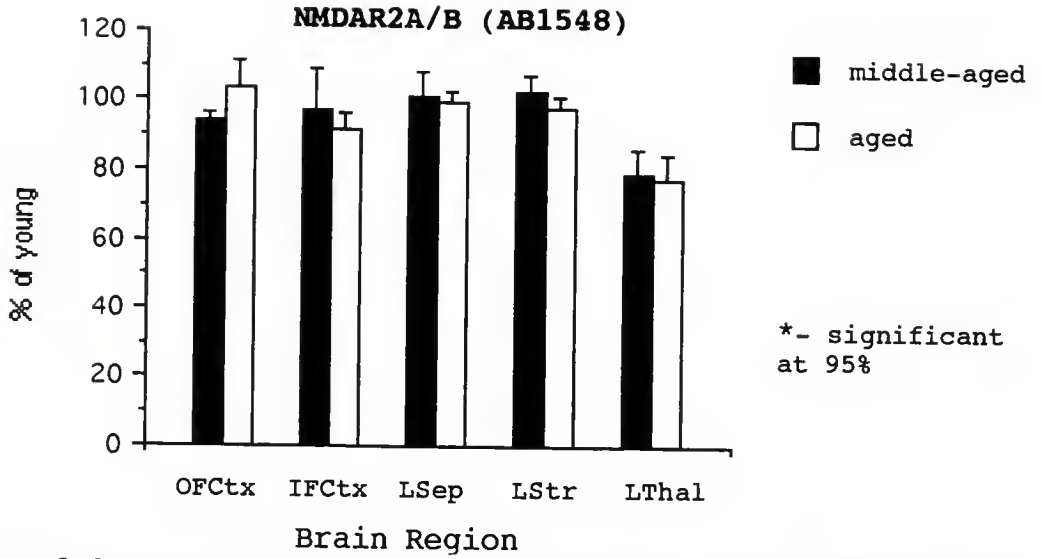


Figure 6-3. Cortical and subcortical brain regions that did not undergo a significant age-related decrease in NMDAR2A/B antibody staining density. (OFCtx= outer frontal cortex; IFCtx= inner frontal cortex; LSep= lateral septum; LStr= lateral striatum; LThal= lateral thalamus). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate. Data are presented as a percentage of young (6-month-old) animals.

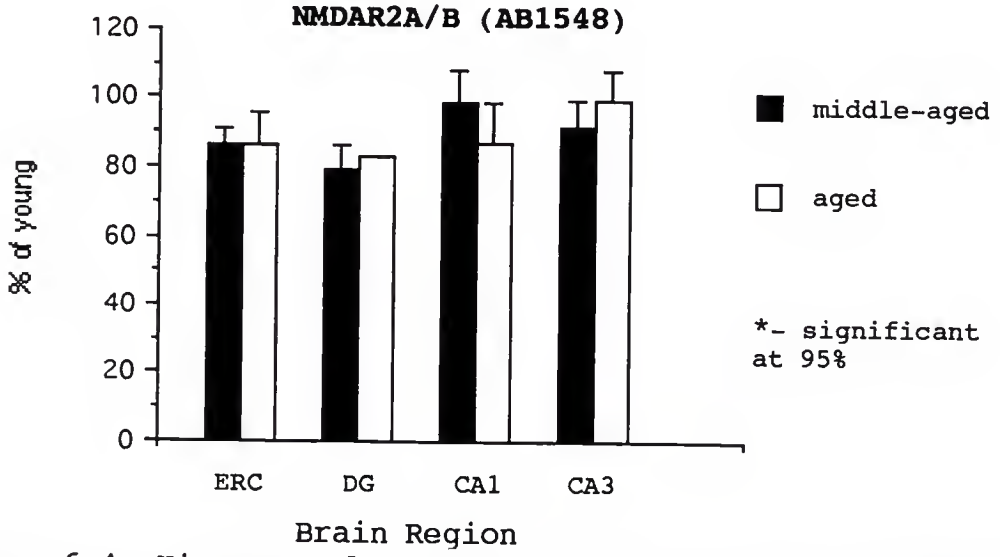
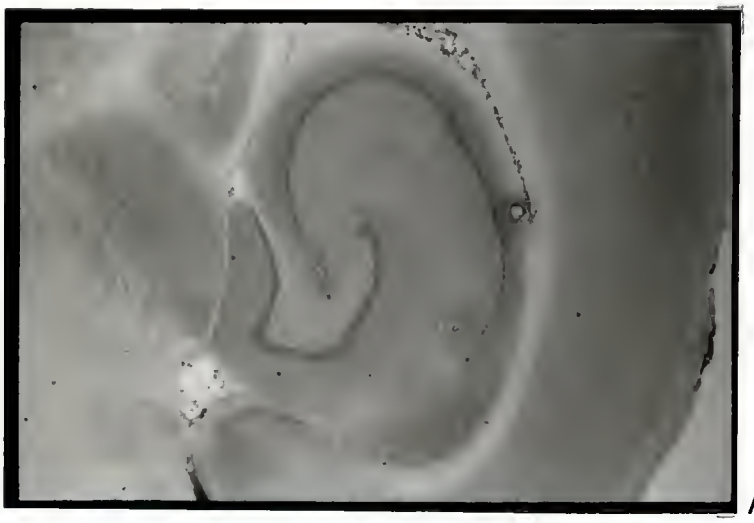
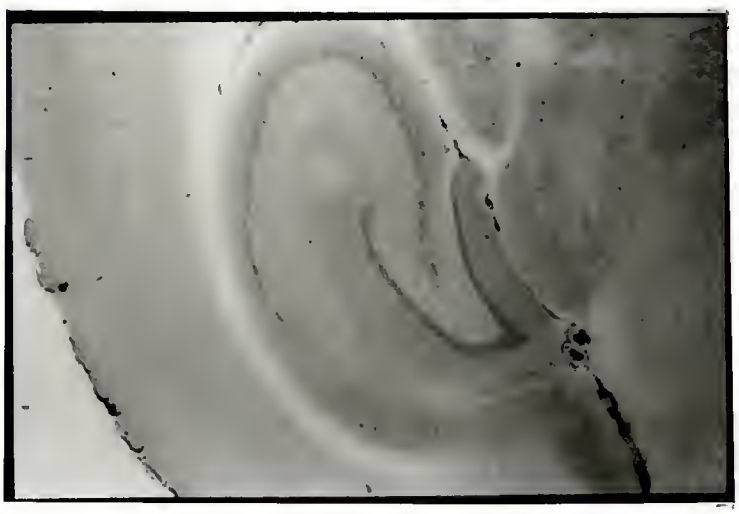


Figure 6-4. Hippocampal and entorhinal cortical brain regions that did not undergo a significant age-related decrease in NMDAR2A/B antibody staining density. (ERC= entorhinal cortex; DG= molecular layer of the dentate gyrus; CA1= hippocampal area CA1; CA3= hippocampal area CA3). Repeated measures ANOVA with between subject randomized blocks and age as a linear covariate. Data are presented as a percentage of young (6-month-old) animals.

Figure 6-5. Representative brain section showing A) NMDAR1 (AB1516) immunostaining ; and B) NMDAR2A/B (AB1548) immunostaining in the hippocampus. Note the identical distribution of both antisera. This overlapping distribution was seen in all brain regions analyzed.



A



B

Discussion

A significant age-related decrease in mRNA levels coding for the NMDAR1 subunit of the NMDA receptor was demonstrated in Chapter 5. However, no concomitant decrease in NMDAR1 protein immunostaining was found. This discrepancy may be because relative levels of mRNA for an NMDA receptor subunit in a neuron may not always coincide with expression of the protein. For example, a recent study using Northern blots demonstrated that mRNA for the NMDAR1 subunit is abundantly expressed in PC12 cells yet, no functional NMDA-operated channels were found (Sucher et al., 1993). Furthermore, immunodetection with a monoclonal antibody indicated the presence of only trace amounts of NMDAR1 protein.

Post-transcriptional mechanisms may be important in the expression of NMDAR1 protein. It was shown that there is a significant decrease in NMDAR1 mRNA levels in specific brain regions of aging rats (Chapter 5) without a loss of NMDAR1 protein. This may be due, in part, to alterations in some aspect of post-transcriptional mechanisms as a function of age. The thresholds for synaptic activation of transcription factors in the hippocampus and their correlation with long-term potentiation (an *in vitro* model of memory function) has been described by Worley et al. (1993). The transcription factor responses to two stimulus patterns were shown to be blocked by the noncompetitive NMDA receptor antagonist MK-801. There were identical transcription factor responses

observed in adult (6- to 12-month-old) and aged (23 to 26-month-old) Fischer 344 rats. This study therefore suggested that the synaptic mechanisms involved in these responses are preserved in aged animals. Therefore, the discrepancies seen between NMDAR1 mRNA levels and NMDAR1 protein levels may be due to differences in post-transcriptional or translational mechanisms.

The cellular control of NMDAR1 translation into protein and the subsequent expression of functional NMDA-gated channels could occur at several levels. Several studies have suggested that post-transcriptional control of NMDA receptor expression occurs in the CNS. Adult rat cerebellar Purkinje cells were shown to express NMDAR1 mRNA (Moriyoshi et al., 1991), however functional NMDA receptor channels were only demonstrated during early stages of development (Rosenmund et al., 1992). In the peripheral nervous system, NMDAR1 mRNA was shown to be expressed in adult dorsal root ganglia with erratic expression of functional NMDA receptors in dorsal root ganglion cells (Huettner 1990).

It can be speculated that there may be an upregulation of translational activity in aged rats to compensate for their decreased levels of NMDAR1 mRNA. This upregulation may explain why there is no age-dependent difference in NMDAR1 protein levels. Alternatively, NMDAR1 protein may be subject to modification or decreased degradation due to post-translational mechanisms.

A significant decrease in NMDA receptors in the entorhinal cortex, lateral striatum and inner frontal cortex, utilizing [^{3}H]MK-801 binding assays, was shown in Chapter 3. However, no change in NMDAR1 immunoreactivity was seen these same brain regions. One possibility for this discrepancy may be the presence of many receptor molecules in the cytoplasm of cells that bear few receptor molecules on their plasma membrane (Petralia et al., 1994a). Only those NMDA receptor molecules on the plasma membrane, exposed to extracellular ligands, would be able to be identified in ligand binding studies.

No age-related differences in NMDAR1 or NMDAR2A/B protein levels in the rat CNS suggests that the function of the NMDA receptor, rather than the number of binding sites may be responsible for age-related cognitive and memory deficits. In support of this idea, there is a significant age-related decrease in the efficacy with which L-glutamate can maximally enhance [^{3}H]MK-801 binding (Chapter 4). Others have shown that NMDA mediated responses decrease with age in the hippocampus, cortex and striatum (Gonzales et al., 1991).

It is important to note that gene expression can be regulated at each step in the pathway from DNA to RNA to protein (Alberts et al., 1989). Thus, a cell can control the proteins it makes by 1) controlling when and how often a given gene is transcribed (transcriptional control), 2) controlling how the primary RNA transcript is spliced or otherwise processed (RNA processing control), 3) selecting

which completed mRNAs in the cell nucleus are exported to the cytoplasm (RNA transport control), 4) selecting which mRNAs in the cytoplasm are translated by ribosomes (translational control), 5) selectively destabilizing certain mRNA molecules in the cytoplasm (mRNA degradation control), or 6) selectively activating, inactivating, or compartmentalizing specific protein molecules after they have been made (protein activity control) (Alberts et al., 1989). There may be age-related changes occurring at one or more of these aforementioned steps leading to differences in the production of fully functional NMDA receptor/channel complexes.

Therefore, when examining age-related changes that occur in the NMDA receptor, [^{3}H]MK-801 binding analyses may be a more accurate method than *in situ* hybridization (NMDA mRNA) or immunostaining (NMDA antibodies) studies. [^{3}H]MK-801 binding analyses (Chapter 3 and 4) labels NMDA receptors that are in the open or activated state ('functional'). Therefore, [^{3}H]MK-801 binding may more accurately determine the number of NMDA receptors that are responsible for NMDA receptor-mediated neurotransmission as a function of age.

In conclusion, age-related cognitive and memory changes attributed to the NMDA receptor channel/complex may be the result of both a decreased number of available NMDA receptors as well as a decreased functional capacity of the remaining NMDA receptors.

CHAPTER 7
SUMMARY AND DISCUSSION

Research Summary

A significant amount of evidence has accumulated suggesting that a reduction in NMDA receptor-mediated neurotransmission may underlie age-related changes in several forms of neuronal plasticity, including spatial learning (Morris et al., 1986) and long-term potentiation (Collingridge and Bliss, 1987; Danysz et al., 1988). One means by which NMDA neurotransmission could be reduced in aged animals is by an alteration in the NMDA receptor/channel complex itself.

The central hypothesis tested in this research was that there are measurable, anatomically specific aging-related changes in the NMDA receptor and its individual subunits. Within the framework of this general hypothesis are the following specific hypotheses which were all critically addressed in specific chapters:

Hypothesis #1 (Chapter 3): There are age-related differences in the density of the NMDA receptor in brains of 6-, 12- and 24-month-old F-344 rats. *In vitro* quantitative autoradiography was performed on young, middle-aged and aged F-344 rat brains using [^{3}H]MK-801.

Results: The entorhinal cortex (ERC), lateral striatum (LSTR) and the inner frontal cortex (IFCTX) showed a significant decrease in [^{3}H]MK-801 binding in aged animals when compared to young.

Summary: The ERC, IFCTX, and LSTR are brain regions that appear to be among the most vulnerable to the effects of aging (Chapter 3; Magnusson and Cotman, 1993; Serra et al., 1994). These regions are implicated in normal memory function and plasticity of the CNS (DeToledo-Morrell et al., 1984; Gonzales et al., 1991; Petit, 1988). A decrease in NMDA receptors in these specific regions, as a function of age, suggests that some of the cellular mechanisms encoding memory may be impaired.

Hypothesis #2 (Chapter 4): There are age-related differences in the ability of L-glutamate to enhance [^{3}H]MK-801 binding to NMDA receptor channels in brains of 6-, 12- and 24-month-old F-344 rats. Various concentrations of L-glutamate were used to stimulate MK-801 binding within the NMDA receptor/channel complex. The ability of L-glutamate to maximally enhance [^{3}H]MK-801 binding was determined as a function of age. Age-related comparisons were made between dose-response curve generated E_{max} and EC_{50} values.

Results: All brain regions analyzed (i.e., OFCTX, IFCTX, ERC, LSTR, LTHAL, LSEP, DG, and CA1) showed an age-dependent decrease in their E_{max} values without any change in EC_{50} values. The ERC, IFCTX, and LSTR showed the greatest percent decrease in E_{max} values.

Summary: The decrease in L-glutamate's efficacy in stimulating [^{3}H]MK-801 binding may account for some of the age-related decreases in learning and memory processes in the CNS. Down-regulation of NMDA-receptor mediated neurotransmission results in a decrease in responsiveness of the NMDA receptor. This down-regulation may be a compensatory mechanism that ultimately compromises NMDA receptor function yet may provide protection from potential excitotoxic processes.

Hypothesis #3 (Chapter 5): There are selective changes in mRNA coding for subunits of the NMDA receptor in the brains of aged F-344 rats. *In situ* hybridization analyses were performed to determine the density of individual mRNA species coding for NMDA subunits. The density and anatomical distribution of the mRNA for the various subunits (i.e. NMDAR1, NMDAR2A, NMDAR2B, NMDAR2C, NMDAR2D and four alternatively spliced versions of NMDAR1) were determined using brains from young (6-month), middle-aged (12-month) and aged (24-month) F-344 male rats. The density of each subunit mRNA was evaluated as a function of increasing age.

Results: NMDAR1 mRNA levels decreased in all brain regions analyzed (i.e. OFCTX, IFCTX, ERC, LSTR, LTHAL, LSEP, DG, CA1 and CA3) as a function of age. The NMDAR1 splice variant $NR1_{0xx}$ showed an age-dependent decrease in the ERC and the CA3 region of the hippocampus in 24-month-old rats. There was an increase in this same isoform in 12-month-old hippocampal area CA1. $NR1_{1xx}$ mRNA levels increased in 12-

month-old animals' LSEP. $NR1_{x1x}$ mRNA levels increased in 12-month-old rats' LSEP, LSTR and LTHAL. The remaining NMDAR1 subunit mRNA levels did not change as a function of age.

Summary: NMDA receptor subtypes with distinct properties may subserve different functional assignments in neurons (Monyer et al., 1994). One likely molecular mechanism for functional changes may be the regulation of subunit expression. A progressive age-dependent increase in one NMDA receptor subunit's expression with a concomitant decline in the expression of another subunit may account for modifications in NMDA receptor activity and vice-versa. Changes in the levels of NMDA subunit mRNA expression may be controlled by age-dependent genetic programs that ultimately effect the make-up of the NMDA receptor/channel. Changes in mRNA may therefore account for some of the deficits in NMDA receptor-mediated learning and memory processes as a function of age.

Hypothesis #4 (Chapter 6): There are specific age-related changes in NMDAR1 and NMDAR2A/B protein density levels in the rat CNS. Immunocytochemistry determined the localization of the NMDA receptor protein within each neuron in each region of interest in the rat CNS. Any potential changes in NMDAR1 and/or NMDAR2A/B protein density levels associated with increasing age were semi-quantitated using the currently available polyclonal antibodies.

Results: There were no significant age-related differences seen in NMDAR1 or NMDAR2A/B immunostaining levels in any of the brain regions analyzed.

Summary: There may be a significant amount of non-membrane bound (cytosolic) NMDA receptor protein detected by immunostaining that is not involved in the formation of heteromeric NMDA receptor/channel complexes. Only surface-presented NMDA receptors would be directly involved in NMDA receptor-mediated neurotransmission. There may be an overproduction of NMDA receptor protein in the cytoplasm that do not form functional complexes on the membrane surface. Therefore, NMDAR1 and NMDAR2A/B immunohistochemistry may not have selectively determined the number of NMDA receptors that are directly involved in NMDA-mediated neurotranmission processes.

Discussion

Taken together, these results indicate that there were measurable, anatomically specific aging-related changes in the NMDA receptor and its individual subunits. The decrease in [^{3}H]MK-801 binding confirmed that there are age-dependent changes in the number of NMDA receptors in the aged rat brain. These differences may be attributable, in part, to selective changes in specific mRNA coding for the NMDA receptor as seen in Chapter 5. The decrease in E_{max} value seen throughout the CNS of 12- and 24-month-old rats suggests that there are some differences in the efficacy of L-

glutamate to enhance [^{3}H]MK-801 binding to the NMDA receptor/channel complex in aged rats.

A decrease in cognitive processes will occur in a significant number of aged individuals (65 years of age or older). Aged men and women are estimated to comprise 12.7% of the total United States population and 18.4% of Florida's population (U.S. Bureau of the Census, Wash., D.C. 1995). Because of their increasing numbers, a considerable amount of emphasis should be placed on improving the overall quality of life for elderly individuals. Possible areas of improvement must focus on the documented cognitive deficits associated with advancing age (Barnes, 1979; DeToledo-Morrell et al., 1984). Specific targeting of the NMDA receptor using drugs that interact directly with this receptor's binding and modulatory sites has begun, and cognitive improvements have been seen in aged individuals (Cohen and Muller, 1992; Davis et al., 1993; Fiore and Rampello, 1989) (see Chapter 3 discussion). The data obtained from NMDA mRNA analyses may, in the future, allow researchers to genetically manipulate the NMDA receptor. The use of antisense oligodeoxy-nucleotides (ODNs) specific to NMDA receptor subunits may be useful tools for the dissection of the participation of specific molecules in the expression of functional NMDA receptors. The antisense ODNs would suppress translation of specific neuronal molecules and their effect on NMDA receptor function could be examined as a function of age. The characterization of the NMDA receptor using ligand binding

assays (Chapter 3 and 4), *in situ* hybridization (Chapter 5) and immunohistochemistry (Chapter 6) may help to pinpoint the changes occurring within this receptor/channel complex as a function of age. The elucidation of NMDA receptor-specific changes found in these experiments may help to explain the NMDA receptor's involvement in learning and memory deficits associated with advancing age.

LIST OF REFERENCES

Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., Watson, J.D. (1989). Control of gene expression. In Molecular Biology of the Cell., (ed. Robertson, M.) p. 551-553, Garland Publishing, Inc., New York.

Amaral, D.G., and Witter, M.P. (1989). The three-dimensional organization of the hippocampal formation: a review of anatomical data. Neurosci., 31, 571-591.

Anatharam, V., Panchal, R.G., Wilson, A., Kolchine, V.V., Treistman, S.N., and Bayley H. (1992). Combinatorial RNA splicing alters the surface charge on the NMDA receptor. F.E.B.S., 305, 27-30.

Anderson, K.J., Monaghan, D.T. and Geddes, J.W. (1989). NMDA receptors, aging and Alzheimer's Disease. In Novel Approaches to the Treatment of Alzheimer's Disease. (ed. Meyer, E.M., Simpkins, J.W. & Yamamoto, J.), p.293-302, Plenum Press, New York.

Barnard, E.A., Darlison, M.G., and Seeburg, P. (1987). Molecular biology of the $GABA_A$ receptor: the receptor/channel superfamily. Trends Neurosci., 10, 502-508.

Barnes, C.A. (1979). Memory deficits associated with senescence: a neurophysiological and behavioral study in the rat. J. Comp. Physiol. Psychol., 93, 74-104.

Barnes, C.A. and McNaughton, B.L. (1985). An age comparison of the rates of acquisition and forgetting of spatial information in relation to long-term enhancement of hippocampal synapses. Behav. Neurosci., 99, 1040-1048.

Bliss, T.V.P., and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of anaesthetized rabbit following stimulation of the perforant path. J. Physiol., 232, 331-356.

Bonhaus, D.W., Perry, W.B., and McNamara, J.O. (1990). Decreased density, not number, of N-methyl-D-aspartate, glycine and phencyclidine binding sites in hippocampus of senescent rats. Brain Res., 532, 82-86.

Buller, A.L., Larson, H.C., Schneider, B.E., Beaton, J.A., Morrisett, R.A., and Monaghan, D.T. (1994). The molecular basis of NMDA receptor subtypes: native receptor diversity is predicted by subunit composition. J. Neurosci., 14, 5471-5484.

Buller, A.L., Morrisett, R.A., and Monaghan D.T. (1993). The NR2 subunit contributes to the pharmacological diversity of native NMDA receptors. Society for Neuroscience Abstracts, 19, #557.10.

Burnett, D.M., and Zahniser, N.R. (1989). Region-specific loss of a1-adrenergic receptors in rat brain with aging: A quantitative autoradiographic study. Synapse, 4, 143-155.

Cajal, S.R. (1911). Histologie du Systeme Nerveux de l' Homme et des Vertebres (Paris: Maloine), Vol 11, 993.

Cherubini, E., Herrling, P.L., Lanfumey, L., and Stanzione, P. (1988). Excitatory amino acids in synaptic excitation of rat striatal neurones *in vitro*. J. Physiol., 400, 677-690.

Choi, D.W. (1987). Ionic dependence of glutamate neurotoxicity. J. Neurosci., 7, 369-379.

Cohen, S.A., and Muller, W.E. (1992). Age-related alterations of NMDA-receptor properties in the mouse forebrain: partial restoration by chronic phosphatidylserine treatment. Br. Res., 584, 174-180.

Cohen, S.A., and Muller, W.E. (1993). Effects of piracetam on N-methyl-D-aspartate receptor properties in the aged mouse brain. Pharmacol., 47, 217-222.

Coleman, P.D., Flood, D.G., and West, M.J. (1987). Volumes of the components of the hippocampus in the aging F-344 rat. J. Comp. Neurol., 266, 300-306.

Collingridge, G.L. (1987). The role of NMDA receptors in learning and memory. Nature, 330, 604-608.

Collingridge, G.L., and Bliss, T.V.P. (1987). NMDA receptors-their role in long term potentiation. Trends Neurosci., 7, 288-293.

Collingridge, G.L., Kehl, S.J., and McLennan, H. (1983). Excitatory amino acids in synaptic transmission in the Schaffer collateral-commissural pathway of the rat hippocampus. J. Physiol., 334, 33-46.

Cotman, C.W., and Iversen, L.L. (1987). Excitatory amino acids in the brain- focus on NMDA receptors. Trends Neurosci., 10, 263-265.

Danysz, W., Wroblewski, J.T., and Costa, E. (1988). Learning impairment in rats by N-methyl-D-aspartate receptor antagonists. Neuropharmacol., 27, 653-656.

Davis, J.L., Markowska, A.L., Wenk, G.L., and Barnes, C.A. (1993). Acetyl-L-carnitine: behavioral, electrophysiological, and neurochemical effects. Neurobiol. Aging, 14, 107-115.

DeToledo-Morrell, L., and Morrell, F. (1985). Electrophysiological marker of aging and memory loss in rat. Ann. NY Acad. Sci., 444, 296-311.

DeToledo-Morrell, L., Morrell, F., and Fleming, S. (1984). Age-dependent deficits in spatial memory are related to impaired hippocampal kindling. Behav. Neurosci., 98, 902-907.

Durand, G.M., Bennett, M.V.L., and Zukin, R.S. (1993). Splice variants of the N-methyl-D-aspartate receptor NR1 identify domains involved in the regulation by polyamines and protein kinase C. P.N.A.S., 90, 6731-6735.

Durand, G.M., Gregor, P., Zheng, X., Bennett, M.V.L., Uhl, G.R., and Zukin R.S. (1992). Cloning of an apparent splice variant of the rat N-methyl-D-aspartate receptor NMDAR1 with altered sensitivity to polyamines and activators of protein kinase C. P.N.A.S., 89, 9359-9363.

Errington, M.L., Lynch, M.A., and Bliss, T.V.P. (1987). Long-term potentiation in the dentate gyrus: induction and increased glutamate release are blocked by D(-)amino-phosphonovalerate. Neurosci., 20, 279-284.

Fagg, G.E., and Foster, A.C. (1983). Amino acid transmitters and their pathways in the mammalian CNS. Neurosci., 9, 701-719.

Fiore, L., and Rampello, L. (1989). L-Acetylcarnitine attenuates the age-dependent decrease of NMDA-sensitive glutamate receptors in rat hippocampus. Acta Neurol., 11, 346-351.

Flood, J.F., Morley, J.E., and Lanthorn, T.H. (1992). Effect on memory processing by D-cycloserine, an agonist of the NMDA/glycine receptor. Eur. J. Pharmacol., 221, 249-254.

Fonnum, F. (1984). Glutamate: a neurotransmitter in mammalian brain. J. Neurochem., 42, 1-11.

Foster, A.C., and Fagg, G.E. (1984). Acidic amino acid binding sites in mammalian neuronal membranes: their characteristics and relationship to synaptic receptors. Brain Res. Rev., 7, 103-164.

Foster, A.C., and Wong, E.F.H. (1987). The novel anticonvulsant MK-801 binds to the activated state of the N-methyl-D-aspartate receptor. Br. J. Pharmacol., 91, 403-409.

Freeman, G.B., and Gibson, G.E. (1987). Selective alteration of mouse brain neurotransmitter release with age. Neurobiol. Aging, 8, 147-152.

Geinisman, Y., Bondareff, W., and Dodge, J.T. (1977). Partial deafferentation of neurons in the dentate gyrus of the senescent rat. Brain Res., 134, 541-545.

Gonzales, R.A., Brown, L.M., Jones, T.W., Trent, R.B., Westbrook, S.T., and Leslie, S.W. (1991). N-methyl-D-aspartate mediated responses decrease with age in Fischer 344 rat brain. Neurobiol. Aging, 12, 219-225.

Gundersen, H.J.G. (1986). Stereology of arbitrary particles. J. Microscopy, 143, 3-45.

Harris, E.W., Ganong, A.H., and Cotman, C.W. (1984). Long-term potentiation in the hippocampus involves activation of N-methyl-D-aspartate receptors. Brain Res., 323, 132-137.

Hebb, D.O. (1949). The Organization of Behavior., John Wiley & Sons, Inc., New York.

Hennegriff, M., Bahr, B.A., Hall, R.A., Guthrie, K.M., Yamamoto, R.S., Kessler, M., Gall, C.M., and Lynch, G. (1992). Antibodies to the GluR-A, GluR-B/C, GluR-D and NMDA-R1 glutamate receptor subunits: Western blot studies. Society for Neuroscience Abstracts, 18, #45.4.

Hollenberg, M.D. (1985). Biochemical mechanisms of receptor regulation. TIPS, 2, 299-302.

Hollmann, M., O'Shea-Greenfield, A., Rogers, S.W., and Heinemann, S. (1989). Cloning by functional expression of a member of the glutamate receptor family. Nature, 342, 643-648.

Hollmann, M., Boulter, J., Maron, C., Beasley, L., Sullivan, J., Pecht, G., and Heinemann, S. (1993). Zinc potentiates agonist-induced currents at certain splice variants of the NMDA receptor. Neuron, 10, 943-954.

Hollmann, M., Maron, C., and Heinemann, S. (1994). N-glycosylation site tagging suggests a three transmembrane domain topology for the glutamate receptor GluR1. Neuron, 13, 1331-1343.

Huettner, J.E. (1990). Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. Neuron, 5, 255-66.

Huettner, J.E., and Bean, B.P. (1988). Block of N-methyl-D-aspartate-activated current by the anticonvulsant MK-801: selective binding to open channels. P.N.A.S., 85, 1307-1311.

Ingram, D.K., Garafalo, P., Spangler, E.L., Mantione, C.R., Odano, I., and London, E.D. (1992). Reduced density of NMDA receptors and increased sensitivity to dizocilpine-induced learning impairment in aged rats. Brain Res., 580, 273-280.

Ingram, D.K., Spangler, E.L., Iijima, S., Ikari, H., Kuo, H., Greig, N.H., and London, E.D. (1994). Rodent models of memory dysfunction in Alzheimer's disease and normal aging: moving beyond the cholinergic hypothesis. Life Sci., 55, 2037-2049.

Jakoi, E.R., Sombati, S., Gerwin, C., and DeLorenzo, R.J. (1992). Excitatory amino acid receptor activation produces a selective and long-lasting modulation of gene expression in hippocampal neurons. Br. Res., 582, 282-290.

Johnson, J.W., and Ascher, P. (1987). Glycine potentiates the NMDA response in cultured mouse brain neurons. Nature, 325, 529-531.

Kitamura, Y., Zhao, X-H, Ohnuki, T., Takei, M., and Nomura, Y. (1992). Age-related changes in the transmitter glutamate and NMDA receptor/channels in the brain of senescence-accelerated mouse. Neurosci. Lett., 137, 169-172.

Kutsuwada, T., Kashiwabuchi, N., Mori, H., Sakimura, K., Kushiya, E., Araki, K., Meguro, H., Masaki, H., Kumanishi, T., Arakawa, M., and Mishina, M. (1992).

Molecular diversity of the NMDA receptor channel. Nature, 358, 36-41.

Lerma, J. (1992). Spermine regulates N-methyl-D-aspartate receptor desensitization. Neuron, 8, 343-352.

Levisohn, L.F., and Isacson, O. (1991). Excitotoxic lesions of the rat entorhinal cortex. Effects of selective neuronal damage on acquisition and retention of a non-spatial reference memory task. Br. Res., 564, 230-244.

Lindsey, J.D., Landfield, P.W., and Lynch, G. (1979). Early onset and topographical distribution of hypertrophied astrocytes in hippocampus of aging rats. A quantitative study. J.Geront., 34, 661-671.

Magnusson, K.R., and Cotman, C.W. (1993). Age-related changes in excitatory amino acid receptors in two mouse strains. Neurobiol. Aging, 14, 197-206.

Matsumoto, H., Kikuchi, S., and Ito, M. (1982). Age-related changes in glutamate metabolism of cerebral cortical slices fron rats. Neurochem. Res., 7, 679-685.

McGurk, J.F., Bennett, M.L.V., and Zukin, R.S. (1990). Polyamines potentiate responses of N-methyl-D-aspartate receptors expressed in *Xenopus* oocytes. P.N.A.S., 87, 9971-9974.

Meguro, H., Mori, H., Araki, K., Kushiya, E., Kutsuwada, T., Yamazaki, M., Kumanishi, T., Arakawa, M., Sakimura, K., and Mishina, M. (1992). Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. Nature, 357, 70-74.

Miller, J.A., Curella, P., and Zahniser, N.R. (1988). A new densitometric procedure to measure protein levels in tissue slices used in quantitative autoradiography. Brain Res., 447, 60-66.

Monaghan, D.T. (1991). Differential stimulation of [^{3}H]MK-801 binding to subpopulations of NMDA receptors. Neurosci. Lett., 122, 21-24.

Monaghan, D.T., and Anderson, K.J. (1991). Heterogeneity and organization of excitatory amino acid receptors and transporters. In Excitatory Amino Acids and Synaptic Transmission, (ed. Wheal, H.V., and Thomson, A.M.) p.33-54, Academic Press Inc., California.

Monaghan, D.T., and Buller, A.L. (1994). Anatomical, pharmacological, and molecular diversity of native NMDA receptor subtypes. In The NMDA Receptor, 2nd edition. (ed. Watkins, J.C., and Collingridge, G.L.) Oxford Univ. Press, New York.

Monaghan, D.T., Clark, H.C., and Schneider, B.E. (1993). Distributions of NMDA receptor subtypes correspond to specific receptor subunits. Society for Neuroscience Abstracts 19, #557.9.

Monaghan, D.T., and Cotman, C.W. (1985). Distribution of N-methyl-D-aspartate-sensitive L-[^{3}H]glutamate-binding sites in rat brain. J.Neurosci., 5, 2909-2919.

Monaghan, D.T., Olverman, H.G., Nguyen, L., Watkins, J.C., & Cotman, C.W. (1988). Two classes of N-methyl-D-aspartate recognition sites: differential distribution and differential regulation by glycine. P.N.A.S., 85, 9836-9840.

Monyer, H., Sprengel, R., Schoepfer, R., Herb, A., Higuchi, M., Lomeli, H., Burnashev, N., Sakmann, B., and Seeburg, P.H. (1992). Heteromeric NMDA receptors: molecular and functional distinction of subtypes. Science, 256, 1217-1221.

Moriyoshi, K., Masu, M., Ishii, T., Shigemoto, R., Mizuno, N., and Nakanishi, S. (1991). Molecular cloning and characterization of the rat NMDA receptor. Nature, 354, 31-37.

Morris, R.G.M., Anderson, E., Lynch, G.S., and Baudry, M.(1986). Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist, AP5. Nature, 319, 774-776.

Morris, R.G.M., Garrard, P., Rawlins, J.N.P., and O'Keefe, J. (1978). Place navigation impaired in rats with hippocampal lesions. Nature, 297, 681-683.

Nakanishi, S. (1992). Molecular diversity of glutamate receptors and implications for brain function. Science, 258, 597-603.

Nakanishi, N., Axel, R., and Schneider, N.A. (1992). Alternative splicing generates functionally distinct N-methyl-D-aspartate receptors, P.N.A.S., 89, 8552-8556.

Nicoll, R.A., Kauer, J.A., and Malenka, R.C. (1988). The current excitement in long-term potentiation. Neuron, 1, 97-103.

Oster, Y., and Schramm, M. (1993). Down-regulation of NMDA receptor activity by NMDA. Neurosci. Lett., 163, 85-88.

Paxinos, G., and Watson, C. (1982). The Rat Brain in Stereotaxic Coordinates. Academic Press Inc., New York.

Peterson, C., and Cotman, C.W. (1989). Strain-dependent decrease in glutamate binding to the N-methyl-D-aspartic acid receptor during aging. Neurosci. Lett., 104, 309-313.

Petit, T.L. (1988). The neurobiology of learning and memory: elucidation of the mechanisms of cognitive dysfunction. Neurotox., 9, 413-428.

Petralia, R.S., Yokotani, N., and Wenthold, R.J. (1994a). Light and electron microscope distribution of the NMDA receptor subunit NMDAR1 in the rat nervous system using a selective anti-peptide antibody. J. Neurosci., 14, 667-696.

Petralia, R.S., Wang, Y.X., and Wenthold, R.J. (1994b). The NMDA receptor subunits NR2A and NR2B show histological and ultrastructural localization patterns similar to those for NR1. J. Neurosci., 14, 6102-6120.

Piggott, M.A., Perry, E.K., Perry, R.H., and Court, J.A. (1992). [^{3}H]MK-801 binding to the NMDA receptor complex, and its modulation in human frontal cortex during development and aging. Br.Res., 588, 277-286.

Pittaluga A., Fedele, E., Risiglione, C., and Raiteri, M. (1993). Age-related decrease of the NMDA-receptor-mediated noradrenaline release in rat hippocampus and partial restoration by D-cycloserine. Eur. J. Pharmacol., 231, 129-134.

Price, M.T., Olney, J.W., and Haft, R. (1981). Age-related changes in glutamate concentration and synaptosomal glutamate uptake in adult rat striatum. Life Sci., 28, 1365-1370.

Quartermain, D., Nuygen, T., Sheu, J., and Herting, R.L. (1991). Milacemaide enhances memory storage and alleviates spontaneous forgetting in mice. Pharmacol. Biochem. Behav., 39, 31-35.

Ransom, R.W., and Deschenes, N.L. (1990). Polyamines regulate glycine interaction with the N-methyl-D-aspartate receptor. Synapse, 5, 294-298.

Ransom, R.W., and Stec, N.L. (1988). Cooperative modulation of [^{3}H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine and polyamines. J. Neurochem., 51, 830-836.

Reynolds, I.J., Murphy, S.H., and Miller, R.J. (1987). ^{3}H-labeled MK-801 binding to the excitatory amino acid receptor complex from rat brain is enhanced by glycine. P.N.A.S., 84, 7744-7748.

Rosenmund, C., Legendre, P., and Westbrook, G.L. (1992). Expression of NMDA channels on cerebellar Purkinje cells acutely dissociated from newborn rats. J. Neurophysiol., 68, 1901-1905.

Sakurai, S., Cha, J.J., Penney, J.B., and Young, A.B. (1990). Regional distribution and properties of [^{3}H]MK-801 binding sites determined by quantitative autoradiography in rat brain. Neurosci., 40, 533-543.

Serra, M., Ghiani, C.A., Foddi, M.C., Motzo, C., and Biggio, G. (1994). NMDA receptor function is enhanced in the hippocampus of aged rats. Neurochem. Res., 19, 483-487.

Shapiro, M.L., & Caramanos, Z. (1990). NMDA antagonist MK-801 impairs acquisition but not performance of spatial working and reference memory. Psychobiol., 18, 231-243.

Spangler, E.L., Bresnahan, E.L., Garafalo, P., Muth, N.J., Heller, B., and Ingram, D.K. (1991). NMDA receptor channel antagonism by dizocilpine (MK-801) impairs performance of rats in aversively-motivated complex maze tasks. Pharmacol. Biochem. Behav., 40, 949-958.

Sprosen, T.S., and Woodruff, G.N. (1990). Polyamines potentiate NMDA induced whole-cell currents in striatal neurons. European J. Pharmacol., 179, 477-478.

Sucher, N.J., Brose, N., Deitcher, D.L., Awobuluyi, M., Gasic, G.P., Bading, H., Cepko, C.L., Greenberg, M.E., Jahn, R., Heinemann, S.F., and Lipton, S.A.(1993). Expression of endogenous NMDAR1 transcripts without receptor protein suggests post-transcriptional control in PC12 cells. J. Biol. Chem., 268, 22299-22304.

Sugihara, H., Moriyoshi, K., Ishii, T., Masu, M., and Nakanishi, S.(1992). Structures and properties of seven isoforms of the NMDA receptor generated by alternative splicing. Biochem. Biophys. Res. Comm., 185, 826-832.

Tamaru, M., Yoneda, Y., Ogita, K., Shimizu, J., and Nagata, Y. (1991). Age-related decreases of the N-methyl-D-aspartate receptor complex in the rat cerebral cortex and hippocampus. Brain Res., 542, 83-90.

Victorica, J., Clark, A., Machado, A., and Satrustegui, J. (1985). Impairment of glutamate uptake and absence of alterations in the energy-transducing ability of old rat brain mitochondria. Mech. Aging Dev., 29, 255-266.

Wenk, G.L., Walker, L.C., Price, D.L., and Cork, L.C. (1991). Loss of NMDA, but not GABA-A, binding in the brains of aged rats and monkeys. Neurobiol. Aging, 12, 93-98.

West, M.J. (1993). New stereological methods for counting neurons, Neurobiol. Aging, 14, 275-285.

Westbrook, G.L., and Jahr, C.E. (1989). Glutamate receptors in excitatory neurotransmission. Semin. Neurosci., 1, 103-114.

Wheeler, D.D., and Ondo, J.G. (1986). Time course of the aging of the high affinity L-glutamate transporter in rat cortical synaptosomes. Exp. Gerontol., 21, 159-168.

Wisden, W., Morris, B.J., and Hunt, S.P.(c1991). *In situ* hybridization with synthetic DNA probes, In Molecular Neurobiology: A Practical Approach, p.205-226, Oxford Univ. Press, New York.

Wo, Z.G., and Oswald, R.E.(1994). Transmembrane topology of two kainate subunits revealed by N-glycosylation. P.N.A.S., 91, 7154-7158.

Wo, Z.G., and Oswald, R.E. (1995). Unraveling the modular design of glutamate-gated ion channels. Trends in Neurosci., 18, 161-168.

Worley, P.F., Bhat, R.T., Baraban, J.M., Erickson, C.A., McNaughton, B.L., and Barnes, C.A. (1993). Thresholds for synaptic activation of transcription factors in hippocampus: correlation with long-term enhancement. J. Neurosci., 13, 4776-4786.

BIOGRAPHICAL SKETCH

Josephine "Jean" Mitchell was born on January 17,1964 in Shalimar, Florida at Eglin Air Force Base where her family was stationed while her father was an active duty officer/pilot in the USAF. Jean's family moved to several places during her childhood, including Turkey, Ohio and Pennsylvania and then settled in Shalimar, Florida where her father retired from active duty. Jean attended Meigs Junior High School and Choctawhatchee Senior High School where she was a member of the jazz and marching bands and the golf team. She graduated in 1982 with honors. It was in Shalimar that Jean met Thomas "Mitch" Mitchell and later married in June of 1985. Jean attended Ocean County College in Tom's River, New Jersey, and graduated with an associate of arts degree in 1985. Jean and Mitch then moved to Gainesville, Florida where she attended the University of Florida and graduated in 1987 with a bachelor's degree in zoology. In 1989, Jean began work in Kevin J. Anderson's laboratory as a technician and became quite interested in the field of neuroscience and therefore entered the Neuroscience Department at the University of Florida College of Medicine in 1990 to pursue her doctoral studies.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kevin J. Anderson, Chair
Associate Professor of
Neuroscience and
Physiological Sciences

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

A. John MacLennan
Assistant Professor of
Neuroscience

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Joanna Peris
Associate Professor of
Pharmacodynamics

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Thomas Vickroy
Associate Professor
of Veterinary Medicine

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Don Walker
Professor of Neuroscience

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1995

Mohan K Raizada
Dean, College of Medicine

Karen A. Holbrook
Dean, Graduate School